

**TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

1241.16

U.S. APPLICATION NO. (If known, give 37 C.F.R. 1.5)

**09/673198**

INTERNATIONAL APPLICATION NO.

PCT/JP99/01987

INTERNATIONAL FILING DATE

14 April 1999

PRIORITY DATE CLAIMED

14 April 1998

## TITLE OF INVENTION

**A PROCESS FOR PRODUCING ISOPRENOID COMPOUNDS BY MICROORGANISMS AND A METHOD FOR SCREENING COMPOUNDS WITH ANTIBIOTIC OR WEEDING ACTIVITY**

APPLICANT(S) FOR DO/EO/US

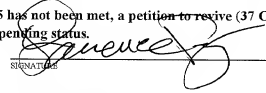
Koichiro Miyake, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time **not later than** delay examination until the expiration of the application time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
  - ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: Submission of Computer Readable Form with paper copy; Form PCT/IS/210 (International Search Report); Form PCT/RO/101 (PCT Request); Copy of published PCT Appl. No. WO99/53071 (first page); Form PCT/IB/308; Inventor Information Sheet

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)		INTERNATIONAL APPLICATION NO.		ATTORNEY'S DOCKET NUMBER	
09/673198		PCT/JP99/01987		1241.16	
17. <input checked="" type="checkbox"/> The following fees are submitted:  <b>Basic National Fee (37 CFR 1.492(a)(1)-(5):</b> Search Report has been prepared by the EP or JPO ..... \$800.00 International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)) ..... \$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)) but international search fee paid to USPTO (37 CFR 1.492(a)(2)) ..... \$710.00 Neither international preliminary examination fee (37 CFR 1.492(a)(1)) nor international search fee (37 CFR 1.492(a)(2)) paid to USPTO ..... \$1,000.00 International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(4)) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00				CALCULATIONS      PTO USE ONLY	
				422 Rec'd PCT/PTO 1 2 OCT 2000	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	27 - 20 =	7	X \$18.00	\$126.00	
Independent Claims	5 - 3 =	2	X \$80.00	\$160.00	
Multiple dependent claim(s) (if applicable)				+ \$270.00	\$270.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$1416.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$	
<b>SUBTOTAL =</b>				\$1416.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$1416.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$40.00	
<b>TOTAL FEES ENCLOSED =</b>				\$1456.00	
<b>Amount to be:</b>					
refunded				\$	
charged				\$	
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>1456.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>06-1205</u> . A duplicate copy of this sheet is enclosed.					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b> <b>SEND ALL CORRESPONDENCE TO:</b>					
Lawrence S. Perry FITZPATRICK, CELLA, HARPER & SCINTO 30 Rockefeller Plaza New York, NY 10112 Tel: (212) 218-2100 Fax: (212) 218-2200			SIGNATURE  _____ Lawrence S. Perry NAME		
			31,865 REGISTRATION NUMBER		

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: )  
: Examiner: Not Yet Assigned  
KOICHIRO MIYAKE, ET AL. )  
: Group Art Unit: N/Y/A  
Application No.: N/Y/A )  
:   
Filed: Currently herewith )  
:   
For: A PROCESS FOR PRODUCING )  
ISOPRENOID COMPOUNDS BY :  
MICROORGANISMS AND A )  
METHOD FOR SCREENING :  
COMPOUNDS WITH )  
ANTIBIOTIC OR WEEDING :  
ACTIVITY ) October 11, 2000

Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Prior to action on the merits, please amend the  
above-identified application as follows:

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 10, lines 9-18, replace "Using the collected  
DNA fragments, ... in *E. coli* K12, can be used." with --For  
cloning the amplified DNA obtained above, an appropriate  
cloning vector is digested with restriction enzymes creating  
the cohesive ends which are able to ligate with the  
amplified DNA fragment. Using a recombinant DNA obtained by

ligating the above amplified DNA with the cloning vector, *Escherichia coli*, e.g., *E coli* DH5  $\alpha$  (available from TOYOBO CO., LTD.) is transformed.

As a cloning vector for cloning the amplified DNA, any cloning vectors including phage vectors and plasmic vectors, which can autonomously replicate in *E coli* K12, can be used--.

Page 35, line 10, before "were detected", insert --As a result of screening by colony hybridization using the DIG-labeled DNA fragment as a probe, which had been obtained by the above method, two types of DNA fragments--.

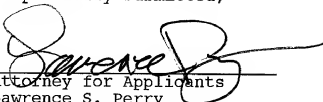
#### REMARKS

The specification has been amended to correct inadvertent translational errors. No new matter has been added.

Entry hereof is earnestly solicited.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,

  
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# SPECIFICATION

## A Process for Producing Isoprenoid Compounds by Microorganisms and A Method for Screening Compounds with Antibiotic or Weeding Activity

### Technical Field

The present invention relates to a method for producing isoprenoid compounds using a transformant derived from a prokaryote; and a method for screening substances having antibiotic or weeding activity involved in a non-mevalonate pathway.

### Background Art

Isoprenoid is a general term for compounds having isoprene unit consisting of 5 carbon atoms as a backbone structure. Isoprenoid is biosynthesized by polymerization of isopentenyl pyrophosphate (IPP). Various kinds of isoprenoid compounds are present in nature and many of them are useful for humans.

For example, ubiquinone plays an important role *in vivo* as an essential component of the electron transport system. The demand for ubiquinone is increasing not only as a pharmaceutical effective against cardiac diseases, but also as a health food in Western countries.

Vitamin K<sub>1</sub>, an important vitamin involved in the blood coagulation system, is utilized as a hemostatic agent. Recently it has been suggested that vitamin K is involved in osteo-metabolism, and is expected to be applied to the treatment of osteoporosis. Phylloquinone and menaquinone have been approved as pharmaceuticals.

In addition, ubiquinone and vitamin K are effective in inhibiting barnacles from clinging to objects, and so would make an excellent additive to paint products to prevent barnacles from clinging.

Further, compounds called carotenoids having an isoprene backbone consisting of 40 carbon atoms have antioxidant effect. Carotenoids such as  $\beta$ -carotene, astaxanthin, and cryptoxanthin are expected to possess cancer preventing and immunopotentiating activity.

As described above, isoprenoid compounds include many effective substances. Establishment of an economical process for producing these substances will be a huge benefit to the medical world and

society.

The process for producing isoprenoid compounds through fermentation has already been examined, and examination of culture conditions, strain breeding by mutagenesis, and improvement of yield by genetic engineering techniques have been tested. However, the practical results are limited to individual types of compounds, and there is no known method effective for the isoprenoid compounds in general.

Isopentenyl pyrophosphate (IPP), a backbone unit of isoprenoid compounds, has been proved to be biosynthesized from acetyl-CoA via mevalonic acid (mevalonate pathway) in eukaryotes, such as an animal and yeast.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is considered to be a rate-limiting enzyme in the mevalonate pathway [Mol. Biol. Cell, **5**, 655 (1994)]. A test in yeast to improve the yield of carotenoids by overexpression of HMG-CoA reductase has been conducted [Misawa, et al., Summaries of Lectures on Carotenoids, 1997].

There is no knowledge which proves the presence of the mevalonate pathway in prokaryotes. In many prokaryotes, another pathway, the non-mevalonate pathway, has been found in which IPP is biosynthesized via 1-deoxy-D-xylulose 5-phosphate produced by condensation of pyruvic acid and glyceraldehyde 3-phosphate [Biochem. J., **295**, 517 (1993)]. It is suggested that 1-deoxy-D-xylulose 5-phosphate is converted to IPP via 2-C-methyl-D-erythritol 4-phosphate in an experiment using <sup>13</sup>C-labelled substrate [Tetrahedron Lett. **38**, 4769 (1997)].

In *Escherichia coli*, a gene encoding an enzyme, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) which allows biosynthesis of 1-deoxy-D-xylulose 5-phosphate by condensation of pyruvic acid and glyceraldehyde 3-phosphate, is identified [Proc. Natl. Acad. Sci. USA, **94**, 12857 (1997)]. Said gene is contained in an operon consisting of four ORFs that include *ispA* encoding farnesyl pyrophosphate synthase.

Further in *Escherichia coli*, the presence of the activity to convert 1-deoxy-D-xylulose 5-phosphate to 2-C-methyl-D-erythritol 4-phosphate is known [Tetrahedron Lett. **39**, 4509 (1998)].

At present there are no known description nor suggestion to improve yield of an isoprenoid compound by genetically engineering these genes contained in the operon.

Although knowledge about the non-mevalonate pathway in prokaryotes has gradually increased,

most enzymes involved therein and genes encoding these enzymes still remain unknown.

In photosynthetic bacteria, there is a known process for effectively producing ubiquinone-10 by introducing a gene for an enzyme ubiC (ubiC gene), which converts chorismate into 4-hydroxybenzoate, and a gene for p-hydroxybenzoate transferase (ubiA) (Japanese Unexamined Patent Application 107789/96). However, there is no example which improved the productivity of isoprenoid compounds by genetically engineering genes for enzymes involved in the non-mevalonate pathway.

Moreover, there is no knowledge about how prokaryotes will be influenced when the reaction on the non-mevalonate pathway is inhibited by mutagenesis or treating with drugs.

#### Disclosure of the Invention

The object of this invention is to provide a process for producing isoprenoid compounds comprising integrating DNA into a vector wherein the DNA contains one or more DNA involved in biosynthesis of isoprenoid compounds useful in pharmaceuticals for cardiac diseases, osteoporosis, homeostasis, prevention of cancer, and immunopotential, health food and anti-fouling paint products against barnacles, introducing the resultant recombinant DNA into a host cell derived from prokaryotes, culturing the obtained transformant in a medium, allowing the transformant to produce and accumulate isoprenoid compounds in the culture, and recovering the isoprenoid compounds from said culture; a process for producing proteins comprising integrating DNA into a vector wherein the DNA contains one or more DNA encoding a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds, introducing the resultant recombinant DNA into a host cell, culturing the obtained transformant in a medium, allowing the transformant to produce and accumulate said protein in the culture, and recovering said protein from the culture; the protein; and DNA encoding the protein. A further object of this invention is to provide a method of screening a substance having antibiotic and/or weeding activities, which comprises screening the substance inhibiting enzymatic reaction on the non-mevalonic acid pathway.

The inventors have completed the invention by finding that the productivity of isoprenoid can be improved by screening DNA capable of improving the productivity for isoprenoid in prokaryotes, and introducing the obtained DNA into prokaryotes.

That is, the first invention of the present application is a process for producing isoprenoid



compounds comprising integrating DNA into a vector wherein the DNA contains one or more DNA selected from the following (a), (b), (c), (d), (e) and (f):

- (a) a DNA encoding a protein having activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate,
- (b) a DNA encoding farnesyl pyrophosphate synthase,
- (c) a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:3, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 3 and has activity to improve efficiency in the biosynthesis of isoprenoid compounds,
- (d) a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:4, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 4 and has activity to improve efficiency in the biosynthesis of isoprenoid compounds,
- (e) a DNA encoding a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate, and
- (f) a DNA encoding a protein that can hybridize under stringent conditions with DNA selected from (a), (b), (c), (d) and (e), and has activity substantially identical with that of the protein encoded by the selected DNA;

introducing the resultant recombinant DNA into a host cell derived from prokaryotes, culturing the obtained transformant in a medium; allowing the transformant to produce and accumulate isoprenoid compounds in the culture; and recovering the isoprenoid compounds from the culture.

Deletions, substitutions or additions of amino acid residues in this specification can be carried out by site-directed mutagenesis, which is a technique well-known prior to the filing of this application. Further, the phrase "one to several amino acid residues" means the number of amino acid residues, which can be deleted, substituted, or added by site-directed mutagenesis, for example, 1 to 5 amino acid residues.

The protein consisting of an amino acid sequence, which has deletion, substitution or addition of one to several amino acid residues, can be prepared according to the methods described in Molecular Cloning: A Laboratory Manual, Second Edition, ed. Sambrook, Fritsch, and Maniatis, Cold Spring

Harbor Laboratory Press, 1989 (hereinafter referred to as Molecular Cloning, Second Edition), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997), Nucleic Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci., USA, 79, 6409 (1982), Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985), and Proc. Natl. Acad. Sci USA, 82, 488 (1985), etc.

The above-mentioned DNA encoding a protein, which catalyzes a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate, is for example, a DNA encoding a protein, which has an amino acid sequence of SEQ ID NO:1, 26 or 28, or a DNA encoding a protein which has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1, 26, or 28 and has activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate.

Examples of such a DNA include a DNA having an nucleotide sequence of SEQ ID NO:6 or a DNA having a nucleotide sequence of SEQ ID NO:27 or 29.

Examples of a DNA encoding farnesyl pyrophosphate synthase include a DNA encoding a protein having an amino acid sequence of SEQ ID NO:2 or a DNA encoding a protein, which has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 2 and has enzymatic activity to produce farnesyl pyrophosphate. A specific example is a DNA having a nucleotide sequence of SEQ ID NO:7.

A specific example of the DNA encoding a protein having an amino acid sequence of SEQ ID NO:3 is a DNA having a nucleotide sequence of SEQ ID NO:8.

Further a specific example of the DNA encoding a protein having an amino acid sequence of SEQ ID NO:4 is a DNA having a nucleotide sequence of SEQ ID NO:9.

Examples of the DNA encoding a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate include a DNA encoding a protein, which has an amino acid sequence of SEQ ID NO:5 or 30, or a DNA encoding a protein, which has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5 or 30 and has activity to catalyze the reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.

Specifically, such a DNA is one having a nucleotide sequence of SEQ ID NO:10 or 31.

The above phrase "DNA...that can hybridize under stringent conditions" means a DNA that can be obtained by colony hybridization, plaque hybridization, Southern Blotting or the like using the above DNA or fragments of the DNA as a probe. Such a DNA can be identified by performing hybridization using a filter with colony- or plaque-derived DNA, or fragments of the DNA immobilized thereon, in the presence of 0.7 to 1.0 mol/l NaCl at 65°C, followed by washing the filter using about 0.1 to 2-fold SSC solution (the composition of SSC solution at 1-fold concentration is consisted of 150mol/l sodium chloride, 15mol/l sodium citrate) at 65°C.

Hybridization can be carried out according to the methods described in Molecular Cloning, Second Edition. Examples of DNA capable of hybridizing include a DNA that shares at least 70% or more homology, preferably, 90% or more homology with a nucleotide sequence selected from SEQ ID NOS:1, 2, 3, 4, and 5.

Examples of isoprenoid compounds include ubiquinone, vitamin K<sub>2</sub>, and carotenoids.

The second invention of this application is a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds and selected from the following (a), (b) and (c):

- (a) a protein having an amino acid sequence of SEQ ID NO:3, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 3
- (b) a protein having an amino acid sequence of SEQ ID NO:4, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 4, and
- (c) a protein having an amino acid sequence of SEQ ID NO:5, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5.

The third invention of this application is a process for producing a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds comprising integrating DNA encoding the protein described in the second invention above into a vector, introducing the resultant recombinant DNA into a host cell, culturing the obtained transformant in a medium, allowing the transformant to produce and accumulate the protein in the culture, and recovering the protein from the culture.

The transformants above include microorganisms belonging to the genus *Escherichia*,

*Rhodobacter* or *Erwinia*.

The fourth invention of this application is a DNA encoding a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds selected from the following (a), (b), (c), (d), (e), (f) and (g):

- (a) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:3,
- (b) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:4,
- (c) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:5,
- (d) a DNA having a nucleotide sequence of SEQ ID NO:8,
- (e) a DNA having a nucleotide sequence of SEQ ID NO:9,
- (f) a DNA having a nucleotide sequence of SEQ ID NO:10, and
- (g) a DNA that can hybridize with any one of DNA described in (a) to (f) under stringent conditions.

The fifth invention of this application is a method for screening a substance having antibiotic activity comprising screening a substance that inhibits the reaction of a protein having activity of an enzyme selected from those present on the non-mevalonate pathway in which 1-deoxy-D-xylulose 5-phosphate biosynthesized from pyruvic acid and glyceraldehyde 3-phosphate is converted to 2-C-methyl-D-erythritol 4-phosphate from which isopentenyl pyrophosphate is biosynthesized.

The sixth invention of this application is a method for screening a substance having weeding activity comprising screening a substance that inhibits the reaction of a protein having activity of an enzyme selected from those present on the non-mevalonate pathway in which 1-deoxy-D-xylulose 5-phosphate biosynthesized from pyruvic acid and glyceraldehyde 3-phosphate is converted to 2-C-methyl-D-erythritol 4-phosphate from which isopentenyl pyrophosphate is biosynthesized.

Examples of the proteins in the fifth and sixth inventions above include a protein of the following (a) or (b):

- (a) a protein having activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate, or
- (b) a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.

Examples of the proteins catalyzing the reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate include a protein having an amino acid sequence of

SEQ ID NO:1, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1, and having activity to catalyze 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate.

Examples of the proteins having activity to catalyze the reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate include a protein having an amino acid sequence of SEQ ID NO:5, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5, and having activity to catalyze the reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.

The seventh invention of this invention is a substance, which has antibiotic activity and is obtained by the screening method in the fifth invention above. Known substances obtained by the above screening method are not included in this invention.

The inventors have focused on structural similarity of fosmidomycin [3-(N-formyl-N-hydroxyamino)propylphosphonic acid] to 2-C-methyl-D-erythritol 4-phosphate, a reaction product from 1-deoxy-D-xylulose 5-phosphate reductoisomerase reaction, or a reaction intermediate assumed to be produced in this enzymatic reaction.

Based on the assumption that fosmidomycin has activity to inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase and antibiotic activity, the inventors have conducted experiments on the screening method of the fifth invention and also described in the following Example 10. As a result, the inventors found that fosmidomycin is a substance having the activity to inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase and antibiotic activity, and in addition, verified the adequacy of the screening method of the fifth invention above. However, known compound fosmidomycin is excluded from this invention.

The eighth invention of this invention is a substance, which has weeding activity and obtained through the screening method of the sixth invention above. As described above, any substance that is obtained from the screening method and already known is excluded from this invention.

Hereinafter a more detailed explanation of this invention will be given.

## I. Cloning of DNA Encoding a Protein Involved in Biosynthesis of Isoprenoid Compounds

(1) Cloning of DNA Encoding a Protein Involved in Biosynthesis of Isoprenoid Compounds using a

## Nucleotide Sequence of DNA (DXS gene) Encoding DXS

Using information on previously-determined nucleotide sequences of *E.coli* chromosome and DXS gene [Proc. Natl. Acad. Sci. USA., 94, 12857 (1997)], a DNA region containing DXS gene or genes neighboring DXS gene is obtained by cloning with PCR method from *E.coli* [Science, 230, 1350 (1985)].

An example of information on a nucleotide sequence containing DXS gene is the nucleotide sequence of SEQ ID NO:11.

A concrete example of methods for cloning the DNA region containing DXS gene is as follows.

*Escherichia coli*, such as an *E.coli* XL1-Blue strain (available from TOYOBO CO., LTD.), is cultured in a suitable medium for *Escherichia coli*, for example, LB liquid medium [containing 10g of Bactotrypton (manufactured by Difco Laboratories), 5g of Yeast extracts (manufactured by Difco Laboratories), 5g of NaCl per liter of water, and adjusted to pH 7.2] according to standard techniques.

After culturing, cells were recovered from the culture by centrifugation.

Chromosomal DNA is isolated from the obtained cells according to a known method, described in, for example, Molecular Cloning, Second Edition.

Using information on a nucleotide sequence of SEQ ID NO:11, a sense primer and an antisense primer, which contain DXS gene or a nucleotide sequence corresponding to the DNA region of genes neighboring DXS gene, are synthesized with a DNA synthesizer.

To introduce the amplified DNA fragments into a plasmid after amplification with PCR, it is preferable to add recognition sites appropriate for restriction enzymes, e.g., *Bam*HI, and *Eco*RI to the 5' ends of sense and antisense primers.

Examples of a combination of the sense and antisense primers include a DNA having a combination of nucleotide sequences: SEQ ID NOS: 12 and 13, SEQ ID NOS: 14 and 15, SEQ ID NOS: 12 and 16, SEQ ID NOS: 17 and 18, SEQ ID NOS: 19 and 13, or SEQ ID NOS: 22 and 23.

Using the chromosomal DNA as a template, PCR is carried out with DNA Thermal Cycler (manufactured by Perkin Elmer Instruments, Inc. Japan) using the primers; TaKaRa LA-PCR™ Kit Ver. 2 (manufactured by TAKARA SHUZO CO., LTD.) or Expand™ High-Fidelity PCR System (manufactured by Boehringer Mannheim K.K.)

In a reaction condition for PCR, PCR is carried out by 30 cycles, in the case of amplifying a DNA fragment of 2kb or less, one cycle consisting of reaction at 94°C for 30 seconds, 55°C for 30 seconds to 1 minute, and 72°C for 2 minutes; in the case of amplifying a DNA fragment of more than 2kb, one cycle consisting of reaction at 98°C for 20 seconds, and 68°C for 3 minutes; then followed by the reaction at 72°C for 7 minutes.

The amplified DNA fragments are cut at sites the same as the restriction enzyme sites added to the above primers, and are fractionated and collected by using agarose gel electrophoresis, sucrose density-gradient centrifugation and the like.

Using the collected DNA fragments, cloning vectors are constructed by standard techniques, such as those described in Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, Supplement 1 to 38, John Wiley & Sons (1987 - 1997), DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995); or by using commercially available kits, such as SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (manufactured by Life Technologies) or a ZAP-cDNA Synthesis Kit (manufactured by Stratagene). Then *Escherichia coli*, e.g., an *E.coli* DH5  $\alpha$  (available from TOYOBO CO., LTD) is transformed using the above cloning vectors.

For transformation of the *E.coli*, any cloning vectors including phage vectors and plasmid vectors, which can autonomously replicate in *E.coli* K12, can be used. Expression vectors for *E.coli* can be used as cloning vectors. Concrete examples of the cloning vectors include ZAP Express [manufactured by Stratagene, Strategies, 5, 58 (1992)], pBluescript II SK(+) [Nucleic Acids Research, 17, 9494 (1989)], Lambda ZAP II (manufactured by Stratagene),  $\lambda$  gt10,  $\lambda$  gt11 (DNA Cloning, A Practical Approach, 1, 49 (1985)),  $\lambda$  TriplEx (manufactured by Clontec),  $\lambda$  ExCell (manufactured by Pharmacia), pT7T318U (manufactured by Pharmacia), pCD2 [H. Okayama and P. Berg; Mol. Cell. Biol., 3, 280 (1983)], pMW218 (manufactured by WAKO PURE CHEMICAL INDUSTRIES., LTD), pUC118 (manufactured by TAKARA SHUZO CO., LTD.), pEG400 [J. Bac., 172, 2392 (1990)], and pQE-30 (manufactured by Qiagen. Inc).

A plasmid DNA containing a DNA of interest can be obtained from the resultant transformant according to standard techniques, such as those described in Molecular Cloning, Second Edition, Current Protocols in Molecular Biology, Supplement 1 to 38, John Wiley & Sons (1987 - 1997), DNA Cloning

1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995).

A plasmid DNA containing a DNA encoding a protein having activity to catalyze the reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate, a DNA encoding farnesyl pyrophosphate synthase, a DNA encoding a protein having an amino acid sequence of SEQ ID NO:3, a DNA encoding a protein having an amino acid sequence of SEQ ID NO:4 or the like; and a plasmid DNA containing one or more DNAs above, can be obtained by the above methods.

Such plasmids include plasmid pADO-1 that contains all of the DNA above, plasmid pDXS-1 or pQEDXS-1 that contains a DNA having a nucleotide sequence of SEQ ID NO:6, plasmid pISP-1 that contains a DNA having a nucleotide sequence of SEQ ID NO:7, plasmid pXSE-1 that contains a DNA having a nucleotide sequence of SEQ ID NO:8, and plasmid pTFE-1 that contains a DNA having a nucleotide sequence of SEQ ID NO:9.

Using the nucleotide sequences of DNA fragments derived from *E.coli*, which have been inserted into these plasmids, homologues of the DNA can be obtained from other prokaryotes, such as microorganisms belonging to the genus *Rhodobacter*, in the same manner as described above.

(2) Cloning of DNA Encoding a Protein Having Activity to Complement methylerythritol-requiring mutant of *E.coli* (Gene Complementing Methylerythritol-requiring Mutant)

① Construction of *E.coli* methylerythritol-requiring mutant

*Escherichia coli*, such as *E.coli* W3110 (ATCC14948), is cultured according to standard techniques.

After culturing, cells are recovered from the obtained culture by centrifugation.

The obtained cells are washed with an appropriate buffer agent, such as 0.05mol/l Tris-maleate buffer (pH 6.0). Then the cells are suspended in the same buffer such that the cell density is  $10^4$  to  $10^{10}$  cells/ml.

Mutagenesis is carried out by standard techniques using the suspension. In such a standard technique, for example, NTG is added to the suspension to a final concentration of 600mg/l, and then the mixture is maintained for 20 minutes at room temperature.

This suspension after mutagenesis is spread on minimal agar medium supplemented with 0.05 to 0.5% methylerythritol and cultured.

An example of minimal agar medium is M9 medium (Molecular Cloning, Second Edition)



supplemented with agar.

Methylerythritol that is chemically synthesized according to the method described in Tetrahedron Letters, 38, 35, 6184 (1997) may be used.

Colonies grown after culturing are replicated on minimal agar media and minimal agar media each containing 0.05 to 0.5% methylerythritol. The mutant of interest, which requires methylerythritol to grow, is selected. That is, a strain capable of growing on minimal agar media containing methylerythritol but not on minimal agar media lacking methylerythritol is selected.

Strain ME 7 is an example of the resultant methylerythritol-requiring mutant obtained by the above manipulations.

## ② Cloning of the Gene Complementing Methylerythritol-requiring nature

*Escherichia coli*, such as *E. coli* W3110 (ATCC14948), is inoculated into culture media, e.g., LB liquid medium, then cultured to the logarithmic growth phase by standard techniques.

Cells are collected from the resultant culture by centrifugation.

Chromosomal DNA is isolated and purified from the obtained cells according to standard techniques, such as those described in Molecular Cloning, Second Edition. The chromosomal DNA obtained by the method described in (1) above can be used as isolated and purified chromosomal DNA.

An appropriate amount of the chromosomal DNA is partially digested with an appropriate restriction enzyme, such as *Sau* 3 A I. The digested DNA fragments are fractionated by according to standard techniques, such as sucrose density-gradient centrifugation (26,000 rpm, 20°C, 20 hr).

The DNA fragments obtained by the above fractionation, 4 to 6 kb each, are ligated to a vector, e.g., pMW118 (Nippon Gene), which has been digested with an appropriate restriction enzyme to construct a chromosomal DNA library.

The methylerythritol-requiring mutant isolated in ① above, such as the strain ME 7, is transformed using the ligated DNA according to standard techniques, e.g., those described in Molecular Cloning, Second Edition.

The resulting transformants are spread on minimal agar media supplemented with a drug corresponding to a drug-resistant gene carried by the vector, such as M9 agar medium containing 100  $\mu$ g/l of ampicillin, then cultured overnight at 37°C.

Thus, transformants that have recovered their methylerythritol requirement can be selected by the

method above.

Plasmids are extracted from the resultant transformants by standard techniques. Examples of a plasmid that can allow the transformants to recover their methylerythritol requirement are pMEW73 and pQEDXR.

The nucleotide sequence of the DNA integrated into the plasmid is sequenced.

An example of such a nucleotide sequence is a sequence containing a nucleotide sequence for yaeM gene of SEQ ID NO:10. Using the information on the nucleotide sequence for yaeM gene, homologues of yaeM gene can be obtained from other prokaryotes or plants in the same manner as described above.

## II. Production of Proteins having Activity to improve efficiency in the biosynthesis of isoprenoid compounds.

To express the resulting DNA in a host cell, the DNA fragment of interest is digested with restriction enzymes or deoxyribonucleases into one with a proper length containing the gene. Next the fragment is inserted into a downstream of a promoter region in an expression vector. Then the expression vector is introduced into a host cell appropriate for the expression vector.

Any host cell that can express the gene of interest can be used. Examples of the host cell include bacteria belonging to the genera *Escherichia*, *Serratia*, *Corynebacterium*, *Brevibacterium*, *Pseudomonas*, *Bacillus*, *Microbacterium* and the like, yeasts belonging to the genera *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Schwanniomyces*, and the like, animal cells, and insect cells.

Expression vectors used herein can autonomously replicate in the host cell above or be integrated into a chromosomal DNA, and contain a promoter at the position to which the DNA of interest as described above can be transcribed.

When a bacterium is used as a host cell, a preferable expression vector for expression of the DNA above can autonomously replicate in the bacterium and is a recombinant vector comprising a promoter, ribosome binding sequence, the DNA above and a transcription termination sequence. The expression vector may contain a gene to regulate a promoter.

Examples of the expression vector include pBTp2, pBTac1, pBTac2 (all of them are available from Boehringer Mannheim K.K.), pKK233-2 (Pharmacia), pSE280 (Invitrogen), pGEMEX-1 (Promega), pQE-8 (Qiagen. Inc), pQE-30 (Qiagen. Inc), pKYP10 (Japanese Patent Laid Open

Publication No. 58-110600), pKYP200 (Agricultural Biological Chemistry, 48, 669, 1984), pLSA1 (Agric. Biol. Chem., 53, 277, 1989), pGEL1 (Proc. Natl. Acad. Sci. USA, 82, 4306, 1985), pBluescriptII SK+, pBluescriptII SK (-) (Stratagene), pTrs30 (FERM BP-5407), pTrs32 (FERM BP-5408), pGEX (Pharmacia), pET-3 (Novagen), pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTP5, pC194, pUC18 (gene, 33, 103, 1985), pUC19 (Gene, 33, 103, 1985), pSTV28 (TAKARA SHUZO CO., LTD.), pSTV29 (TAKARA SHUZO CO., LTD.), pUC118 (TAKARA SHUZO CO., LTD.), pPA1 (Japanese Patent Laid Open Publication No. 63-233798), pEG400 (J. Bacteriol., 172, 2392, 1990), and pQE-30 (Qiagen, Inc).

Any promoter that can function in a host cell may be used. Examples of such a promoter include promoters derived from *Escherichia coli* or phages, such as *trp* promoter (P *trp*), *lac* promoter (P *lac*), P<sub>L</sub> promoter, P<sub>R</sub> promoter, P<sub>SE</sub> promoter, SP01 promoter, SP02 promoter, and penP promoter. Furthermore, P *trp* x 2 promoter that is formed by joining two P *trp* in series, and *lac* promoter, *letI* promoter, and *lact7* promoter; those artificially designed and modified, can be used.

Any ribosome binding sequence that can function in a host cell can be used. A preferable plasmid has a distance between Shine-Dalgarno sequence and a starting codon appropriately adjusted, of for example 6 to 18 bases long.

A transcription termination sequence is not always required for expression of the DNA of interest. Preferably, a transcription termination sequence is arranged immediately followed by a structural gene.

Examples of the host cell used herein include microorganisms belonging to the genera *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Microbacterium*, *Serratia*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Scenedesmun*, *Streptomyces*, *Synnecoccus*, and *Zymomonas*. Preferable host cells include microorganisms belonging to the genera *Escherichia*, *Corynebacterium*, *Brevibacterium*, *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Chromatium*, *Erwinia*, *Methylobacterium*, *Phormidium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Scenedesmun*, *Streptomyces*, *Synnecoccus* and *Zymomonas*.

More specific examples of the host cell include *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* DH5  $\alpha$ , *Escherichia coli* MC1000, *Escherichia coli*

KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli* NY49, *Escherichia coli* MP347, *Escherichia coli* NM522, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum* ATCC14068, *Brevibacterium saccharolyticum* ATCC14066, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, *Corynebacterium glutamicum* ATCC13032, *Corynebacterium glutamicum* ATCC14297, *Corynebacterium acetoacidophilum* ATCC13870, *Microbacterium ammoniophilum* ATCC15354, *Serratia ficaria*, *Serratia fonticola*, *Serratia liquefaciens*, *Serratia marcescens*, *Pseudomonas* sp. D-0110, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Agrobacterium rubi*, *Anabaena cylindrica*, *Anabaena doliolum*, *Anabaena flos-aquae*, *Arthrobacter aureus*, *Arthrobacter citreus*, *Arthrobacter globiformis*, *Arthrobacter hydrocarbophilus*, *Arthrobacter myxodermus*, *Arthrobacter nicotianae*, *Arthrobacter paraffinicus*, *Arthrobacter protophormiae*, *Arthrobacter roseoparaffinus*, *Arthrobacter sulfireus*, *Arthrobacter ureafaciens*, *Chromatium buderii*, *Chromatium tepidum*, *Chromatium vinosum*, *Chromatium warmingii*, *Chromatium fluviatile*, *Erwinia uredovora*, *Erwinia carotovora*, *Erwinia ananas*, *Erwinia herbicola*, *Erwinia punctata*, *Erwinia terreus*, *Methylobacterium rhodesianum*, *Methylobacterium extorquens*, *Phomidium* sp. ATCC29409, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospseudomonas blastica*, *Rhodospseudomonas marina*, *Rhodospseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodospirillum salexigens*, *Rhodospirillum salinarum*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Streptomyces fungicidicus*, *Streptomyces griseochromogenes*, *Streptomyces griseus*, *Streptomyces lividans*, *Streptomyces olivogriseus*, *Streptomyces rameus*, *Streptomyces tanashiensis*, *Streptomyces vinaceus*, and *Zymomonas mobilis*.

Any method to introduce a recombinant vector into the host cell as described above may be used. Examples of such a method include a method using calcium ions (Proc. Natl. Acad. Sci. USA, 69, 2110, 1972), protoplast method (Japanese Patent Laid Open Publication No. 63-2483942), or methods described in Gene, 17, 107 (1982) or Molecular & General Genetics, 168, 111 (1979).

When yeast is used as a host cell, expression vectors are, for example, YEpl3 (ATCC37115), YEpl24 (ATCC37051), YCp50 (ATCC37419), pHS19, and pHS15.

Any promoter that can function in yeast can be used. Examples of such a promoter include PH05 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock

protein promoter, MF  $\alpha$  1 promoter, and CUP1 promoter.

Host cells used herein include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Trichosporon pullulans*, and *Schwanniomyces alluvius*.

Any method to introduce a recombinant vector, that is, to introduce DNA into yeast may be used. Examples of such methods include Electroporation (Methods. Enzymol., 194, 182, 1990), Spheroplast method (Proc. Natl. Acad. Sci. USA, 75, 1929 (1978)), lithium acetate method (J. Bacteriol., 153, 163 (1983)), and methods described in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978).

When an animal cell is used as a host cell, expression vectors are, for example, pcDNA1, pcDM8 (Funakoshi Co., Ltd), pAGE107 [Japanese Patent Laid Open Publication No. 3-22979; Cytotechnology, 3, 133 (1990)], pAS3-3 [Japanese Patent Laid Open Publication No. 2-227075, pCDM8 (Nature, 329, 840 (1987)), pcDNA1/Amp (Invitrogen), pREP4 (Invitrogen), pAGE103 [J. Biochem., 101, 1307 (1987)], and pAGE210.

Any promoter that can function in an animal cell may be used. Examples of such promoters include a promoter for IE (immediate early) gene of cytomegalovirus (human CMV), SV40 initial promoter, retrovirus promoter, metallothionein promoter, heat shock promoter, and SR  $\alpha$  promoter. Moreover, an enhancer of human CMV IE gene may be used together with a promoter.

Host cells used herein are, for example, Namalwa cells, HBT5637 (Japanese Patent Laid Open Publication No. 63-299), COS1 cells, COS7 cells, and CHO cells.

Any method to introduce a recombinant vector into an animal cell, that is, to introduce DNA into an animal cell may be used. Examples of such methods include Electroporation [Cytotechnology, 3, 133 (1990)], calcium phosphate method (Japanese Patent Laid Open Publication No. 2-227075), lipofection [Proc. Natl. Acad. Sci., USA, 84, 7413 (1987)], and methods described in Virology, 52, 456 (1973). Recovery and culture of the transformant can be carried out according to methods described in Japanese Patent Laid Open Publication No. 2-227075 and Japanese Patent Laid Open Publication No. 2-257891.

When an insect cell is used as a host cell, proteins can be expressed according to methods described in, such as Baculovirus Expression Vectors, A Laboratory Manual, Current Protocols in Molecular Biology Supplement 1-38 (1987-1997), and Bio/Technology, 6, 47 (1988).

That is, a vector for introducing a recombinant gene and Baculovirus are co-transduced into an

insect cell to obtain a recombinant virus in the culture supernatant of the insect cell. Then an insect cell is infected with the recombinant virus, resulting in expression of the protein of interest.

Examples of the vectors to transfer genes include pVL1392, pVL1393, pBlueBacIII (all of which are manufactured by Invitrogen).

Baculoviruses used herein are, for example, Autographa californica nuclear polyhedrosis virus that infects *Barathra* insects.

Examples of the insect cells include ovarian cells of *Spodoptera frugiperda*, Sf9, and Sf21 (Baculovirus Expression Vectors, A Laboratory Manual (W. H. Freeman and Company, New York, 1992), and of *Trichoplusia ni*, High 5 (Invitrogen).

Methods of co-transduction of the vector for transferring the recombinant gene and the Baculovirus into an insect cell to prepare a recombinant virus include calcium phosphate transfection (Japanese Patent Laid Open Publication No. 2-227075) and, lipofection [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)].

Methods for expressing genes include secretory production, and fusion protein expression according to the techniques shown in Molecular Cloning, Second Edition, in addition to direct expression.

When the gene is expressed in yeasts, animal cells, or insect cells, a protein to which sugar or a sugar chain is added, can be obtained.

Proteins having activity to improve efficiency in the biosynthesis of isoprenoid compounds can be produced by culturing a transformant containing a recombinant DNA to which the above DNA has been introduced in a medium, allowing the transformant to produce and accumulate proteins having activity to improve efficiency in the biosynthesis of isoprenoid compounds in the culture, then collecting the proteins from the culture.

The transformants for producing proteins with activity to improve efficiency in the biosynthesis of isoprenoid compounds of the present invention, can be cultured by standard techniques to culture a host cell.

When the transformant of this invention is prokaryote such as *Escherichia coli* or eukaryote such as yeast, a medium for culturing such transformants contains a carbon source, a nitrogen source, and inorganic salts, which the microorganisms can assimilate, and allows the transformant to grow efficiently.

Either natural media or synthetic media can be used if they satisfy the above conditions.

Any carbon source assimilable by the microorganisms may be used. Such carbon sources include glucose, fructose, sucrose, and molasses containing them, carbohydrates e.g., starch or hydrolysates of starch, organic acids e.g., acetic acid and propionic acid, and alcohols e.g., ethanol and propanol.

Examples of nitrogen sources include ammonia, salts of inorganic acids or organic acids, e.g., ammonium chloride, ammonium sulfate, ammonium acetate, and ammonium phosphate, other nitrogen-containing compounds, peptone, meat extract, yeast extract, corn steep liquor, casein hydrolysates, soybean meal and soybean meal hydrolysate, various fermentation microorganic cells or their digests.

Examples of inorganic salts include potassium primary phosphate, potassium secondary phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, and calcium carbonate.

Culturing is carried out by shaking culture or submerged aeration-agitation culture are carried out under aerobic conditions. The preferable culture temperature ranges from 15 to 40°C. The preferable culture period ranges from 16 hours to 7 days. The pH is kept within a range from 3.0 to 9.0 while culturing. The pH is adjusted using inorganic or organic acid, alkaline solutions, urea, calcium carbonate, ammonia or the like.

If necessary, an antibiotics e.g., ampicillin or tetracycline may be added to the media while culturing.

When microorganisms transformed with the expression vectors using inducible promoters are cultured, inducers may be added to the media if necessary. For example, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) or the like may be added to the media when microorganisms transformed with the expression vectors containing lac promoter are cultured; indoleacrylic acid (IAA) or the like may be added when microorganisms transformed with the expression vectors containing trp promoter are cultured.

The media for culturing a transformant obtained by using an animal cell as a host cell include a generally used RPMI1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM medium [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)],

199 medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] or those to which fetal calf serum or the like is added.

Normally, the transformant is cultured in the presence of 5% CO<sub>2</sub> for 1 to 7 days at pH 6 to 8 and 30 to 40°C.

If necessary, antibiotics e.g., kanamycin and penicillin may be added to the medium while culturing.

Examples of media to culture a transformant obtained by using an insect cell as a host cell include a generally used TNM-FH medium (Pharming), Sf-900 II SFM medium (GIBCO BRL), ExCell400, ExCell405 (both manufactured by JRH Biosciences), Grace's Insect Medium (Grace, T.C.C., Nature, 195, 788 (1962)).

The transformant is generally cultured for 1 to 5 days at pH 6 to 7 and at 25°C to 30°C.

If necessary, antibiotics e.g., gentamycin may be added to the medium while culturing.

Proteins having activity to improve efficiency in the biosynthesis of isoprenoid compounds of this invention can be isolated and purified from the culture of the transformant of this invention by standard isolation and purification techniques for an enzyme.

For example, when the protein of this invention is expressed in a soluble form within the cell, after the culture is completed the cells are recovered by centrifugation, suspended in aqueous buffer, then disrupted using an ultrasonicator, french press, Manton Gaulin homogenizer, Dyno-Mill, or the like, thereby obtaining cell-free extracts. The cell-free extract is separated by centrifugation to obtain the supernatant. The purified sample can be obtained from the supernatant by one of or a combination of standard techniques for isolating and purifying enzymes. Such techniques include a solvent extracting technique, salting out technique using ammonium sulfate, desalting technique, precipitation technique using organic solvents, anion exchange chromatography using resins such as diethylaminoethyl (DEAE) - Sepharose, and DIAION HPA-75 (Mitsubishi Chemical Corp.), cation exchange chromatography using resins e.g., S-Sepharose FF (Pharmacia), hydrophobic chromatography using resins e.g., butylsepharose, phenylsepharose, gel filtration using molecular sieve, affinity chromatography, chromatofocusing, and electrophoresis such as isoelectric focusing.

When the proteins that form inclusion bodies are expressed in the cells, the cells are recovered, disrupted, and separated by centrifugation, thereby obtaining precipitated fractions. From



the resulting precipitated fractions, the protein is recovered by standard techniques, and then the insoluble protein is solubilized using a protein denaturing agent. The solubilized solution is diluted or dialyzed to an extent that the solution contains no protein denaturing agent or that the concentration of protein denaturing agent does not denature protein, thereby allowing the protein to form a normal three-dimensional structure. Then the purified sample can be obtained by the same techniques for isolation and purification as described above.

When the protein of this invention or its derivative, such as a sugar-modified protein, is secreted outside the cell, the protein or its derivative, such as a sugar chain adduct, can be recovered from the culture supernatant. That is, the culture is treated by centrifugation and the like as described above so as to obtain soluble fractions. From the soluble fractions, the purified sample can be obtained using the techniques for isolation and purification as described above.

The resulting protein as described above is, for example a protein having an amino acid sequence selected from amino acid sequences of SEQ ID NOS: 1 to 5.

Moreover, the protein expressed by the method above can be chemically synthesized by techniques including Fmoc method (fluorenylmethyloxycarbonyl method), tBoc method (t-butyloxycarbonyl method). Further, the protein can be synthesized by using a peptide synthesizer of Souwa Boeki K.K. (Advanced ChemTech, U.S.A.), Perkin-Elmer Japan (Perkin-Elmer, U.S.A.), Pharmacia BioTech (Pharmacia BioTech, Sweden), ALOKA CO., LTD. (Protein Technology Instrument), KURABO INDUSTRIES LTD. (Synthecell-Vega, U.S.A), PerSeptive Limited., Japan (PerSeptive, U.S.A), or SHIMADZU CORP.

### III. Production of Isoprenoid Compound

Isoprenoid compounds can be produced by culturing the transformants obtained as described in II above according to the method of II above, allowing the transformants to produce and accumulate isoprenoid compounds in the culture, then recovering the isoprenoid compounds from the culture.

The above culture can yield isoprenoid compounds, such as ubiquinone, vitamin K<sub>2</sub>, and carotenoids. Specific examples of isoprenoid compounds include ubiquinone-8 and menaquinone-8 produced using microorganisms belonging to the genus *Escherichia* as a transformant, ubiquinone-10 produced using those belonging to the genus *Rhodobacter*, vitamin K<sub>2</sub> produced using those belonging to the genus *Arthrobacter* as a transformant, astaxanthin produced using those belonging to the genus

*Agrobacterium* as a transformant, and lycopene,  $\beta$ -carotene, and zeaxanthin produced using those belonging to the genus *Erwinia* as a transformant.

After the culture is completed, in order to isolate and purify isoprenoid compounds, isoprenoid compounds are extracted by adding an appropriate solvent to the culture, the precipitate is removed by e.g., centrifugation, and then the product is subjected to various chromatography.

#### IV. Screening a Substance inhibiting Enzymatic Activity on Non-Mevalonate Pathway

##### (1) Determination of Enzymatic Activity on Non-Mevalonate Pathway

The enzymatic activity on non-mevalonate pathway can be determined according to normal methods for determining enzymatic activity.

The pH of the buffer used as a reaction solution to determine activity should be within a range that does not inhibit the enzymatic activity of interest. A preferable pH range includes the optimal pH.

For example, a buffer at pH 5 to 10, preferably 6 to 9 is used for 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

Any buffer can be used herein so far as it does not inhibit the enzymatic activity and can be adjusted to the pH above. Examples of such a buffer include Tris-hydrochloric acid buffer, phosphate buffer, borate buffer, HEPES buffer, MOPS buffer, and bicarbonate buffer. For example, Tris-hydrochloric acid buffer can preferably be used for 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

A buffer of any concentration may be employed so far as it does not inhibit the enzymatic activity. The preferable concentration ranges from 1mol/l to 1mol/l.

When the enzyme of interest requires a coenzyme, a coenzyme is added to the reaction solution. For example, NADPH, NADH or other electron donors can be used as a coenzyme for 1-deoxy-D-xylulose 5-phosphate reductoisomerase. A preferable coenzyme is NADPH.

Any concentration of the coenzyme to be added can be employed so far as it does not inhibit reaction. Such a concentration preferably ranges from 0.01 mol/l to 100 mol/l, more preferably, 0.1 mol/l to 10 mol/l.

Metal ions may be added to a reaction solution if necessary. Any metal ion can be added so far as it does not inhibit reaction. Preferable metal ions include  $\text{Co}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$ .

Metal ions may be added as metallic salts. For example, a chloride, a sulfate, a carbonate, and a phosphate can be added.

Any concentration of the metal ion to be added can be employed so far as it does not inhibit reaction. A preferable concentration ranges from 0 mol/l to 100 mol/l, more preferably, 0.1 mol/l to 10 mol/l.

The substrate of the enzyme of interest is added to the reaction solution. For example, 1-deoxy-D-xylulose 5-phosphate is added for 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

Any concentration of the substrate may be employed so far as it does not inhibit reaction. The preferable concentration ranges from 0.01 mol/l to 0.2 mol/l in the reaction solution.

The enzyme concentration used in reaction is not specifically limited. Normally, the concentration ranges from 0.01 mg/ml to 100 mg/ml.

An enzyme used herein is not necessarily purified into a single substance. It may contain contaminative proteins. In the search as described in (2) below, cellular extracts containing 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity or cells having the same activity can be used.

Any reaction temperature may be employed so far as it does not inhibit enzymatic activity. A preferable temperature range includes the optimal temperature. That is, the reaction temperature ranges from 10°C to 60°C, more preferably, 30°C to 40°C.

Activity can be detected by a method for measuring a decrease in substrates accompanying the reaction or an increase in reaction products as the reaction proceeds.

Such a method is a method wherein the substance of interest is separated and quantitatively determined by e.g. high performance liquid chromatography (HPLC) if necessary. When NADH or NADPH increases or decreases as the reaction proceeds, activity can directly be determined by measuring the absorbance at 340 nm of the reaction solution. For example, the activity of 1-deoxy-D-xylulose 5-phosphate reductoisomerase can be detected by measuring a decrease in the absorbance at 340 nm using a spectrophotometer to determine NADPH quantity that decreases as the reaction proceeds.

## (2) Screening a Substance Inhibiting Enzymatic Activity on the Non-mevalonate pathway

A substance inhibiting enzymatic activity on the non-mevalonate pathway can be screened for by adding the substance to be screened for to the enzymatic activity measurement system as described in (1) above, allowing the mixture to react similarly, and then screening a substance that suppresses the amount of the substrates decreased in comparison to a case when no such substance is added; or a substance that

suppresses the yield of the reaction product.

Screening methods include a method wherein the decrease in the amount of substrates or the increase in the amount of reaction products is traced with time; or a method where after the reaction has proceeded for a certain period the decrease in the amount of substrates or the increase in the amount of reaction products is measured.

In the method wherein the decrease in the amount of substrates or the increase in the amount of reaction products is traced with time, the amount is measured preferably at 15 seconds to 20 minutes intervals, more preferably at 1 to 3 minutes intervals during reaction.

To measure the decrease in the amount of substrates or the increase in the amount of reaction products after reaction has proceeded for a certain period, the reaction period is preferably 10 minutes to 1 day, more preferably, 30 minutes to 2 hours.

A substance inhibiting the enzymatic activity on the non-mevalonate pathway inhibits the growth of microorganisms and plants that possess the non-mevalonate pathway. The inventors have first found the fact that this substance inhibits the growth of the microorganisms and plants.

The non-mevalonate pathway is present in microorganisms and plants, but absent in animals and humans. Therefore, the substance inhibiting the enzymatic activity on the non-mevalonate pathway but not affecting human and animals can be obtained by the above described screening method.

This substance can be an effective antibiotic or herbicide.

This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application Nos. 10-103101, 10-221910 and 11-035739, which are priority documents of the present application.

#### Brief Description of Drawings

Figure 1 shows the effect of reaction temperature on 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity.

Figure 2 shows the effect of the pH of the reaction solution on 1-deoxy-D-xylulose 5-phosphate reductoisomerase activity. Enzymatic activity measured at various pH in 100 mol/l Tris-hydrochloric acid buffer are shown. Activity is shown as a relative activity when activity at pH 8.0 is considered as

100 %.

Figure 3 shows a method for disrupting *yaeM* gene on a chromosome using homologous recombination.

Figure 4 shows the effect of fosmidomycin on 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

#### Best Mode for Carrying Out the Invention

The invention will now be described by way of examples, but shall not be limited thereto. Unless otherwise specified, gene recombination shown in the examples was carried out according to techniques described in Molecular Cloning, Second Edition (hereinafter referred to as the standard techniques).

Example 1 Cloning of DNA encoding proteins involved in the biosynthesis of isoprenoid compounds

(1) Cloning of DNA encoding proteins involved in the biosynthesis of isoprenoid compounds using the nucleotide sequence of *E.coli* DXS gene

One platinum loop of *E.coli* XL1-Blue (purchased from TOYOBO) was inoculated into 10 ml of LB liquid medium, then cultured overnight at 37°C.

After culturing, cells were collected by centrifugation from the resultant culture.

Chromosomal DNA was isolated and purified from the cells according to the standard techniques.

Sense and antisense primers, each having *Bam*H I and *Eco*R I restriction enzyme sites at their 5'-ends and consisting of nucleotide sequence pairs of SEQ ID NOS:12 and 13, 14 and 15, 12 and 16, 17 and 18, and 19 and 13; and sense and antisense primers, each having *Bam*H I restriction enzyme site at their 5'-ends and consisting of a nucleotide sequence pair of SEQ ID NO:22 and 23; were synthesized using a DNA synthesizer.

PCR was carried out with a DNA Thermal Cycler (Perkin Elmer Instruments, Inc. Japan) using these primers, chromosomal DNA as a template, and a TaKaRa La-PCR™ Kit Ver. 2 (TAKARA SHUZO CO., LTD.), Expand™ High-Fidelity PCR System (Boehringer Mannheim K.K.) or a Taq DNA polymerase (Boehringer).

PCR was carried out for 30 cycles. In the case of amplifying a DNA fragment of 2kb or less, one cycle consisting of reaction at 94°C for 30 seconds, 55°C for 30 seconds to 1 minute, and 72°C for

2 minutes; in the case of amplifying a DNA fragment of more than 2kb, one cycle consisting of reaction at 98°C for 20 seconds, and 68°C for 3 minutes; then followed by the reaction at 72°C for 7 minutes.

Among the DNA fragments amplified by PCR, DNA fragments amplified using sense and antisense primers, each having BamH I and EcoR I restriction enzyme sites at their 5'-ends, were digested with restriction enzymes BamH I and EcoR I; DNA fragments amplified using sense and antisense primers, each having BamH I restriction enzyme site at their 5'-ends, were digested with restriction enzyme BamH I.

After the digestion, these DNA fragments treated with the restriction enzymes were subjected to agarose gel electrophoresis and recovered BamH I and EcoR I-treated DNA fragments and BamH I-treated DNA fragments.

A broad host range vector pEG 400 containing lac promoter [J. Bac., 172, 2392 (1990)] was digested with restriction enzymes BamH I and EcoR I, subjected to agarose gel electrophoresis and recovered BamH I and EcoR I-treated pEG 400 fragments.

pUC118 (TAKARA SHUZO CO., LTD.) was digested with a restriction enzyme BamH I, then subjected to agarose gel electrophoresis and recovered BamH I-treated pUC 118 fragments.

Each of the resultant BamH I and EcoR I-treated DNA fragments was mixed with BamH I and EcoR I-treated pEG 400 fragments, then the mixture was allowed to precipitate with ethanol. The obtained DNA precipitate was dissolved in 5  $\mu$ l of distilled water for ligation reaction to occur, thereby obtaining each recombinant DNA.

Using the resultant recombinant DNA, *E.coli* (purchased from TOYOBO) DH5  $\alpha$  was transformed according to the standard techniques. Then the transformant was spread on LB agar medium containing 100  $\mu$ g/ml of spectinomycin, then cultured overnight at 37°C.

Some colonies of the transformant resistant to spectinomycin were cultured in 10 ml of LB liquid medium containing 100  $\mu$ g/ml of spectinomycin with shaking for 16 hours at 37°C.

The resulting culture was centrifuged, so that cells were collected.

Plasmids were isolated from the cells according to the standard techniques.

To confirm that the isolated plasmids contained the DNA fragment of interest, the plasmids were cleaved with various restriction enzymes to examine their structures and their nucleotide sequences were sequenced.

A plasmid containing a DNA with a nucleotide sequence of SEQ ID NO:6, DNA with a nucleotide sequence of SEQ ID NO:7, DNA with a nucleotide sequence of SEQ ID NO: 8, and DNA with a nucleotide sequence of SEQ ID NO: 9 was named pADO-1. A plasmid containing a DNA with a nucleotide sequence of SEQ ID NO: 6 was named pDXS-1. A plasmid containing a DNA with a nucleotide sequence of SEQ ID NO: 7 was named pISP-1. A plasmid containing a DNA with a nucleotide sequence of SEQ ID NO: 9 was named pTFE-1.

The above BamH I-treated DNA fragments and BamH I-treated pUC118 fragments were mixed, then the mixture was allowed to precipitate with ethanol. The resulting DNA precipitate was dissolved in 5  $\mu$ l of distilled water for ligation reaction to occur to obtain recombinant DNA. *Escherichia coli* was transformed using the recombinant DNA in the same manner as described above, then plasmids were isolated from the transformants.

To confirm the isolated plasmids contain the DNA fragments of interest, the plasmids were cleaved with various restriction enzymes to examine their structures and their nucleotide sequences were sequenced in the same manner as described above.

These plasmids were digested with BamH I. The DNA fragments of interest were recovered in the same manner as described above, then sub-cloned into an expression vector pQE30 (Qiagen. Inc).

The plasmid obtained by the sub-cloning above and having a nucleotide sequence of SEQ ID NO:6 was named pQEDXS-1.

## (2) Cloning of the Gene Complementing Methylerythritol-requiring nature

### ① Selection of Methylerythritol-requiring Mutant of *Escherichia coli*

*E.coli* W3110 (ATCC14948) was inoculated into LB liquid medium and cultured to its logarithmic growth phase.

After culturing, cells were recovered from the resulting culture by centrifugation.

The cells were washed with 0.05 mol/l Tris-maleate buffer (pH 6.0), then suspended in the same buffer to the cell density of  $10^9$  cells/ml.

Mutation was induced by adding NTG to the suspension to a final concentration of 600mg/l, and then the mixture was maintained for 20 minutes at room temperature.

These NTG treated cells were spread on M9 minimal agar medium containing 0.1 % methylerythritol (Molecular Cloning, Second Edition) plate and cultured.

Methylerythritol was chemically synthesized according to the method described in Tetrahedron Letters, 38, 35, 6184 (1997).

Colonies grown on M9 minimal agar medium containing 0.1 % methylerythritol were replicated on M9 minimal agar medium and on M9 minimal agar medium containing 0.1 % methylerythritol. The mutant of interest, a strain requiring methylerythritol to grow, was selected. That is, a strain capable of growing on a minimal agar medium containing 0.1 % methylerythritol but not on the same lacking methylerythritol was selected.

The thus obtained methylerythritol-requiring mutant ME7 was used in the following experiments.

## ② Cloning of the Gene Complementing Methylerythritol-requiring nature

*Escherichia coli* W3110 (ATCC14948) was inoculated into LB liquid medium, then cultured to its logarithmic growth phase. Then cells were collected from the resultant culture by centrifugation.

Chromosomal DNA was isolated and purified from the obtained cells according to the standard techniques.

200  $\mu$ g of the chromosomal DNA was partially digested with a restriction enzyme, *Sau* 3AI. The resulting DNA fragments were fractionated by sucrose density-gradient centrifugation (26,000 rpm, 20°C, 20 hr).

The DNA fragments obtained by the above fractionation, 4 to 6 kb each, were ligated to pMW118 vector (Nippon Gene), which had been digested with a restriction enzyme *Bam*H I, constructing a genomic DNA library.

Using this genomic DNA library, the strain ME7 isolated in ① above was transformed according to the standard techniques.

The resulting transformants were spread on LB agar medium supplemented with 100  $\mu$ g/l of ampicillin, then cultured overnight at 37°C.

Plasmids were extracted from each colony that grew on the agar medium and then the nucleotide sequences were determined.

The plasmids determined its nucleotides sequence had contained the nucleotide sequence of SEQ ID NO:10. These plasmids were named pMEW41 and pMEW73.

A plasmid extracted from one strain of the clones having the sequence was named pMEW73.

The pMEW73 was double-digested with *Hind* III and *Sac* I. The resultant *Hind* III and *Sac* I-



treated DNA fragment having a nucleotide sequence of SEQ ID NO:10 was ligated to multi-cloning sites of broad host range vector pEG400 [J. Bac., 172, 2392 (1990)], constructing pEGYM1.

The Hind III - Sac I-treated DNA fragment was ligated to the Hind III - Sac I site of vector pUC19 (Gene, 33, 103 (1985)), constructing pUCYM-1.

According to the information on the nucleotide sequence of chromosomal DNA of *Escherichia coli* based on Genbank data base, the DNA fragment that had been inserted into the vector was confirmed to contain yaeM gene.

A recombinant vector, which can express yaeM gene sufficiently, was constructed by following method with PCR [Science, 230, 1350 (1985)].

A sense primer having a sequence of SEQ ID NO:20 and an antisense primer having a sequence of SEQ ID NO:21 were synthesized using a DNA synthesizer.

A Bam H I restriction enzyme recognition site was added to each 5'-end of the sense and antisense primers.

yaeM gene was amplified by PCR with DNA Thermal Cycler (Perkin Elmer Instruments, Inc. Japan) using chromosomal DNA of *E.coli* as a template, these primers and Taq DNA polymerase (Boehringer).

PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for 30 seconds, reaction at 55°C for 30 seconds, and reaction at 72°C for 2 minutes followed by reaction at 72°C for 7 minutes.

After the amplified DNA fragments and pUC118 (TAKARA SHUZO CO., LTD.) were digested with a restriction enzyme BamH I, each of the DNA fragments were purified by agarose gel electrophoresis.

Both of these fragments were mixed, then the mixture was allowed to precipitate with ethanol. The resultant DNA precipitate was dissolved in 5  $\mu$ l of distilled water for ligation reaction to occur, thereby obtaining recombinant DNA.

The recombinant DNA was confirmed to be yaeM gene by determining the nucleotide sequences, then sub-cloned to expression vector pQE30 (Qiagen, Inc).

The resulting recombinant DNA was named pQEYM1.

The strain ME7 was transformed using pQEYM1 by standard techniques. The transformant

was spread on LB agar medium containing 100  $\mu$ g/ml of ampicillin, then cultured overnight at 37°C.

The transformants were confirmed to form colonies at the same growth rate as wild-type strain, suggesting that yaeM gene complemented mutation in the strain ME7.

#### Example 2 Production of Ubiquinone-8 (CoQ8) using Recombinant *Escherichia coli*

(1) *E.coli* DH5 $\alpha$  were transformed using the plasmids pADO-1, pDXS-1, and pXSE-1, those obtained in Example 1 above, and pEG400 as a control, respectively, then *E.coli* DH5  $\alpha$ /pADO-1, *E.coli* DH5  $\alpha$ /pDXS-1, *E.coli* DH5  $\alpha$ /pXSE-1 and *E.coli* DH5  $\alpha$ /pEG400 that showed resistance to spectinomycin at a concentration of 100  $\mu$ g/ml were obtained

These transformants were inoculated into a test tube containing 10 ml of LB medium supplemented with thiamine and vitamin B<sub>6</sub>, 100 mg/l each, 50 mg/l of p-hydroxybenzoic acid, and 100  $\mu$ g/ml of spectinomycin. Then the transformants were cultured with shaking for 72 hours at 30°C.

After the culture was completed, each culture was concentrated 10 - fold.

To each 300  $\mu$ l of concentrated culture, 300  $\mu$ l 2-butanol and 300  $\mu$ l glass beads were added. Isoprenoid compounds were extracted with the solvent while disrupting the cells by Multi Beads Shocker MB-200 (YASUI KIKAI) for 5 minutes. Then the 2-butanol layer was collected by centrifugation.

The amount of CoQ8 produced by the transformants was calculated by Quantitative analysis of the CoQ8 in the butanol layer using high performance liquid chromatography (LC-10A, SHIMADZU CORP.).

HPLC was carried out using Develosil ODS-HG-5 (NOMURA CHEMICAL K.K.) as a column, and methanol:n-hexane = 8:2 solution as a mobile phase at 1ml/min of the flow rate and 275 nm of the measuring wavelength.

Table 1 shows the results.

Table 1

CoQ8 Production by transformant of *Escherichia coli*

Transformant	Cell Amount (OD660)	Amount of CoQ8 Produced (mg/L)	Intracellular Content *1
<i>E.coli</i> DH5 $\alpha$ /pEG400	5.8	0.63	1.1
<i>E.coli</i> DH5 $\alpha$ /pADO-1	5.5	0.98	1.8
<i>E.coli</i> DH5 $\alpha$ /pDXS-1	5.2	0.85	1.6
<i>E.coli</i> DH5 $\alpha$ /pXSE-1	5.6	0.67	1.2

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production (mg/L) by a cell amount (OD660).

The amount of CoQ8 produced was significantly higher in DH5  $\alpha$ /pADO-1, DH5  $\alpha$ /pDXS-1 and DH5  $\alpha$ /pXSE-1 than in the control strain DH5  $\alpha$ /pEG400. In particular, the highest productivity was shown by DH5  $\alpha$ /pADO-1 to which all DNA obtained in Example 1 were introduced.

(2) *E.coli* DH5  $\alpha$ /pDXS-1 or *E.coli* DH5  $\alpha$ /pEG400, as obtained in (1) above, was inoculated into a test tube containing 10 ml of a M9 medium, and then cultured with shaking for 72 hours at 30°C.

After the culture was completed, the amount of CoQ8 produced by the transformants was calculated in the same manner as in (1) above.

Table 2 shows the results.

Table 2

CoQ8 Production by transformant of *Escherichia coli*

Transformant	Cell amount (OD660)	Amount of CoQ8 Produced (mg/L)	Intracellular Content *1
<i>E.coli</i> DH5 $\alpha$ /pEG400	3.1	0.49	1.6
<i>E.coli</i> DH5 $\alpha$ /pDXS-1	2.5	1.02	4.1

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production (mg/L) by a cell amount (OD660).

The amount of CoQ8 produced was significantly higher in DH5  $\alpha$ /pDXS-1 than in the control strain DH5  $\alpha$ /pEG400.

(3) Production of CoQ8 using Recombinant *Escherichia coli*

The plasmid pEGYM1 obtained in Example 1 or pEG400 as a control was introduced into *E.coli* DH5  $\alpha$  and *E.coli* DH5  $\alpha$ /pEGYM1 and *E.coli* DH5  $\alpha$ /pEG400 that show resistance to spectinomycin at a concentration of 100  $\mu$ g/ml were obtained.

These transformants were inoculated into a test tube containing 10 ml of LB medium supplemented with 1% glucose, 100 mg/l of vitamin B<sub>1</sub>, 100 mg/l of vitamin B<sub>6</sub>, 50 mg/l of p-hydroxybenzoic acid. Then the transformants were cultured with shaking for 72 hours at 30°C.

After the culture was completed, the amount of CoQ8 produced by the transformants was calculated in the same manner as in (1) above.

Table 3 shows the results.

Table 3

CoQ8 Production by transformants of *Escherichia coli*

Transformant	Cell amount (OD660)	Amount of CoQ8 Produced (mg/L)	Intracellular Content <sup>*1</sup>
<i>E.coli</i> DH5 $\alpha$ /pEG400	14.44	0.83	0.57
<i>E.coli</i> DH5 $\alpha$ /pEGYM1	13.12	0.94	0.71

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production (mg/L) by a cell amount (OD660).

The amount of CoQ8 produced was significantly higher in DH5  $\alpha$ /pEGYM1 than in the control strain DH5  $\alpha$ /pEG400.

### Example 3 Production of Menaquinone-8 (MK-8) by Recombinant *Escherichia coli*

(1) The *E.coli* DH5  $\alpha$ /pADO-1 or *E.coli* DH5  $\alpha$ /pEG400, obtained in Example 2 (1), was inoculated into a test tube containing 10 ml of TB medium supplemented with 100  $\mu$ g/ml of spectinomycin, and then cultured with shaking for 72 hours at 30°C. The TB medium had been prepared by dissolving 12 g of bactotrypton (Difco), 24 g of yeast extract (Difco), and 5 g of glycerol into 900 ml of water followed by the addition of 100ml of aqueous solution containing 0.17mol/l KH<sub>2</sub>PO<sub>4</sub> and 0.72mol/l K<sub>2</sub>HPO<sub>4</sub>.

After the culture was completed, MK-8 was quantified in the same quantifying method for CoQ8 as in Example 2 (1), then the amount of MK-8 produced by the transformants was calculated.

Table 4 shows the results.

Table 4

MK-8 Production by transformants of *Escherichia coli*

Transformant	Cell amount (OD660)	Amount of MK-8 Produced (mg/L)	Intracellular Content *1
<i>E.coli</i> DH5 $\alpha$ /pEG400	23.2	1.1	0.46
<i>E.coli</i> DH5 $\alpha$ /pADO-1	23.5	1.8	0.75

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production amount (mg/L) by a cell amount (OD660).

The amount of MK-8 produced was significantly higher in DH5  $\alpha$ /pADO-1 than in the control DH5  $\alpha$ /pEG400.

(2) *E.coli* DH5  $\alpha$ /pDXS-1 or *E.coli* DH5  $\alpha$ /pEG400, obtained in Example 2 (1), was cultured in the same manner in (1) above, then the amount of MK-8 produced by the transformants was calculated.

Table 5 shows the results.

Table 5

Production of MK-8 by transformants of *Escherichia coli*

Transformant	Cell amount (OD660)	Amount of MK-8 Produced (mg/L)	Intracellular Content *1
<i>E.coli</i> DH5 $\alpha$ /pEG400	42.8	2.41	0.56
<i>E.coli</i> DH5 $\alpha$ /pDXS-1	44.0	2.96	0.67

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production (mg/L) by a cell amount (OD660).

The amount of MK-8 produced was significantly higher in DH5  $\alpha$ /pDXS-1 than in the control strain DH5  $\alpha$ /pEG400.

#### Example 4 Production of CoQ8 by recombinant *Erwinia carotovora*

A plasmid pDXS-1 obtained in Example 1 or pEG400 as a control, was introduced into *Erwinia carotovora* IFO-3380, thereby obtaining transformants IFO-3380/pDXS-1 and IFO-3380/pEG400, both of which were resistant to spectinomycin at a concentration of 100  $\mu$ g/ml.

These transformants were inoculated into a test tube containing 10 ml of LB medium supplemented with 100  $\mu$ g/ml of spectinomycin, and then cultured with shaking for 72 hours at 30°C.

After the culture was completed, the amount of CoQ8 produced by the transformants was calculated in the same manner as in Example 2 (1).

Table 6 shows the results.

Table 6

CoQ8 Production by transformants of *Erwinia carotovora*

Transformant	Cell amount (OD660)	Amount of CoQ8 Produced (mg/L)	Intracellular Content *1
IFO-3380/pEG400	1.68	0.26	1.5
IFO-3380/pDXS-1	2.48	0.45	1.8

\*1: Intracellular content is shown with a value obtained by dividing a 10-fold CoQ8 production (mg/L) by a cell amount (OD660).

The amount of CoQ8 produced was significantly higher in IFO-3380/pDXS-1 than in the control strain IFO-3380/pEG400.

#### Example 5 Production of ubiquinone and carotenoids by recombinant *Erwinia uredovora*

The plasmids pUCYM-1, pQEDXS-1, pQEYM-1, obtained in Example 1, or pUC19 and pQE30 as controls were introduced into *Erwinia uredovora* DSM-30080 by electroporation, and then the transformants, *E. uredovora* DSM-30080/pUCYM-1, *E. uredovora* DSM-30080/pQEDXS-1, *E. uredovora* DSM-30080/pQEYM-1, *E. uredovora* DSM-30080/pUC19 and *E. uredovora* DSM-30080/pQE30, which showed resistant to ampicillin at a concentration of 100  $\mu$ g/ml were obtained.

These transformants were inoculated into a test tube containing 10 ml of LB medium supplemented with 100  $\mu$ g/ml of ampicillin, 1 % glucose, vitamin B<sub>1</sub> and vitamin B<sub>6</sub>, 100 mg/l each, and 50 mg/l of p-hydroxybenzoic acid. Then the transformants were cultured by shaking for 72 hours at 30°C.

After the culture was completed, the amount of CoQ8 produced by the transformants was calculated in the same manner as in Example 2 (1).

The produced amount of carotenoid pigments was calculated by detecting the absorbance at 450 nm for the 2-butanol layer using a spectrophotometer in the same manner as in Example 2 (1).

Table 7 shows the results.

Table 7

Production of CoQ8 and Carotenoids by transformants of *E. uredovora*

Transformants	Cell amount	CoQ8		Carotenoids	
	OD660	Production mg/L	Intracellular content ratio Relative value	Production Relative value	Intracellular content ratio Relative value
DSM-30080/pUC19	2.00	1.15	1.0	1.0	1.0
DSM-30080/pUCYM-1	1.88	1.39	1.3	1.5	1.6
DSM-30080/pQE30	2.52	1.29	1.0	1.0	1.0
DSM-30080/pQEYM-1	1.92	1.36	1.4	1.7	2.2
DSM-30080/pQEDXS-1	2.12	3.21	3.0	5.6	6.7

Both CoQ8 production and carotenoid pigment production were significantly higher in DSM-30080/pUCYM-1 than in the control strain DSM-30080/pUC19.

Similarly, both CoQ8 production and carotenoid pigment production were significantly higher in DSM-30080/pQEYM-1 and DSM-30080/pQEDXS-1 than in the control strain DSM-30080/pQE30.

**Example 6** Cloning of the DNA Encoding Proteins Involved in the Biosynthesis of Isoprenoid Compounds from a Photosynthetic Bacterium *Rhodobacter sphaeroides*

#### (1) Cloning of DXS Gene from *R. sphaeroides*

The Genbank database was searched for DXS homologue conserved in other species using the DXS nucleotide sequence found in *E.coli*. As a result, DXS homologues were found in *Haemophilus influenzae* (P45205), *Rhodobacter capsulatus* (P26242), *Bacillus subtilis* (P54523), *Synechocystis* sp. PCC6803 (P73067) and *Mycobacterium tuberculosis* (007184) and the like. Highly conserved amino acid sequences were selected by comparison of these sequences. A nucleotide sequence corresponding to such a conserved amino acid sequence was designed in consideration of the codon usage in *R.sphaeroides*. A DNA fragment having a nucleotide sequence of SEQ ID NO:32 and of SEQ ID NO:33, and a DNA fragment having a nucleotide sequence of SEQ ID NO:34 were synthesized by DNA synthesizer.

PCR was carried out with DNA Thermal Cycler (Perkin Elmer Instruments, Inc. Japan) using chromosomal DNA of *R.sphaeroides* KY4113 (FERM-P4675) as a template, the primers above, and an Expand™ High-Fidelity PCR System (Boehringer Mannheim K.K.).

PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for 40 seconds, reaction at 60°C for 40 seconds, reaction at 72°C for 1 minute, followed by reaction at 72°C for 1

minute, thereby obtaining the DNA fragment of interest. The DNA fragments were DIG-labeled using DIG DNA Labeling Kit (Boehringer Mannheim K.K.).

To obtain the full length DXS gene of *R.sphaeroides*, a genomic DNA library of a strain KY4113 was constructed. The strain KY4113 was cultured overnight in LB medium, extracting the chromosomal DNA. The chromosomal DNA was partially digested with a restriction enzyme *Sau3AI*, and then 4 to 6 kb DNA fragments were purified by sucrose density-gradient centrifugation. The DNA fragments were ligated with *Bam*H I-digested vector pUC19 using a Ligation Pack (Nippon Gene), and *E.coli* DH5  $\alpha$  was transformed using the ligated DNA. The transformants were spread on LB agar medium containing 100  $\mu$ g/ml of ampicillin, thus obtaining about 10,000 colonies. were detected. As a result of sequencing, ORF sharing high degrees of sequence homology with known DXS gene of other species was found from each DNA fragment. An amino acid sequence of SEQ ID NO:26 was named DXS1 and that of SEQ ID NO:27 was named DXS2.

#### (2) Confirmation of Complementarity using *E.coli* DXS Gene-deleted mutant

##### ① Selection of *E.coli* DXS gene-deleted strain

*E.coli* W3110 (ATCC19498) was inoculated into LB liquid medium, and then cultured to its logarithmic growth phase. After culturing, cells were collected from the culture by centrifugation.

The cells were washed with 0.05 mol/l Tris-maleate buffer (pH 6.0) and suspended in the same buffer to a cell density of  $10^9$  cells/ml.

NTG was added to the suspension to a final concentration of 600 mg/l, then the mixture was maintained for 20 minutes at room temperature to induce mutation.

The resultant NTG-treated cells were spread on a M9 minimum agar medium (Molecular Cloning, Second Edition) plate containing 0.1% 1-deoxyxylulose, then cultured. 1-Deoxyxylulose had been chemically synthesized according to the method described in J. C. S. Perkin Trans I, 2131-2137 (1982).

Colonies grew on M9 minimum agar medium containing 0.1% 1-deoxyxylulose were replicated on M9 minimal agar medium and on M9 minimal agar medium containing 0.1 % 1-deoxyxylulose. The mutant of interest, a strain requiring 1-deoxyxylulose to grow, was selected. That is, a strain capable of growing on minimal agar medium containing 1-deoxyxylulose but not on the same medium lacking 1-deoxyxylulose was selected.

The thus selected and obtained mutant was named ME1.



When pDXS-1 was introduced into the strain ME1, deficiency in 1-deoxyxylulose of the strain ME1 was complemented. Therefore the strain ME1 was confirmed to be a strain from which DXS gene was deleted.

### (3) Complementation Studies on DXS1 and DXS2

DNA fragment encoding DXS1 of SEQ ID NO:27 or a DNA fragment encoding DXS2 of SEQ ID NO:29, respectively, both derived from the strain KY4113, was ligated to downstream of the lac promoter of a vector pUC19 respectively to construct recombinant plasmids.

When the constructed plasmids were introduced into the strain ME1, both DXS1 and DXS 2 each complemented the 1-deoxyxylulose-deficiency in the strain ME 1.

Therefore, *R. sphaeroides* was shown to have two genes, DXS1 and DXS2, having activity to catalyze the reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate.

### (4) Cloning of Gene Complementing Methylerythritol-requiring nature derived from *R. sphaeroides*

The *E.coli* Methylerythritol-requiring mutant ME7 obtained in Example 1 (2) ① was inoculated into LB liquid medium containing 0.1% methylerythritol, cultured to its logarithmic growth phase, then centrifuged to collect cells.

The cells were washed twice with 1mol/l HEPES aqueous solution containing 10 % glycerol so as to remove the medium components as far as possible.

Plasmids were extracted from the genomic library of *R. sphaeroides* KY4113 constructed in Example 6 (1). Then the plasmids were introduced into the washed cells by electroporation according to standard techniques.

Next, the cells were spread on LB agar medium containing 100  $\mu$ g/l of ampicillin, then cultured overnight at 37°C.

After picking up the colonies grown on the medium, the colonies were inoculated into LB liquid medium to culture, then plasmids were extracted from the cells cultured.

When the plasmids extracted were introduced again into the strain ME 7, the transformants could grow in a medium lacking methylerythritol. Therefore it was confirmed that the plasmid contained a DNA fragment complementing methylerythritol-requiring nature derived from *R. sphaeroides*.

As a result of sequencing of the nucleotide sequence of the DNA fragment, the DNA sequence of

SEQ ID NO:31 encoding an amino acid sequence that shares high homology with *E.coli* yaeM was found.

#### Example 7 Production of Ubiquinone-10 (CoQ10) by Recombinant Photosynthetic Bacteria

A *glnB* promoter derived from the strain KY4113 was ligated upstream of the DNA fragment DXS1 of SEQ ID NO:27 and DXS2 of SEQ ID NO:29, both obtained in Example 6. Then the product was inserted into a broad host range vector pEG400, thus constructing plasmids. These plasmids were named pRSDX-1 and pRSDX-2, respectively. In addition, *yaeM* and DXS1 were joined in tandem, then the product was ligated downstream of *glnB* promoter, thereby constructing a plasmid. The plasmid was named pRSYMDX1. These plasmids were introduced into *R. sphaeroides* KY4113, respectively, by electroporation (Bio-Rad Laboratories).

Then the cells were spread on LB agar medium containing spectinomycin at a concentration of  $100\ \mu\text{g/ml}$ , then cultured for 3 days at  $30^\circ\text{C}$ .

Next, colonies that grew on the medium were inoculated into LB medium containing spectinomycin at a concentration of  $100\ \mu\text{g/ml}$ , cultured overnight. Then, the cultured cells were collected by centrifugation.

It was confirmed that the cells of each strain contained the introduced plasmid by extracting the plasmids from the cells (Qiagen, Inc). Thus obtained transformants were named KY4113/pRSDX-1, KY4113/pRSDX-2, KY4113/pRSYMDX1 and KY4113/pEG400.

A platinum loop of each transformant was inoculated into a test tube containing 5 ml of seed medium (2 % glucose, 1 % peptone, 1 % yeast extract, 0.5 % NaCl, pH 7.2 adjusted with NaOH) and then cultured for 24 hours at  $30^\circ\text{C}$ .

0.5ml of the resultant culture was inoculated into a test tube containing 5 ml of ubiquinone-10 production medium, then cultured by shaking for 5 days at  $30^\circ\text{C}$ .

The ubiquinone-10 production medium consisted of 4 % blackstrap molasses, 2.7 % glucose, 4 % corn steep liquor, 0.8 % ammonium sulfate, 0.05 % potassium primary phosphate, 0.05 % potassium secondary phosphate, 0.025 % magnesium sulfate heptahydrate, 3 mg/l of ferrous sulfate heptahydrate, 8 mg/l of thiamine, 8 mg/l of nicotinic acid, and 1 ml/l of trace element, had previously been adjusted to pH 9, supplemented with 1 % calcium carbonate, then autoclaved.

Then the amount of CoQ10 produced by the transformants was calculated in the same

manner as in quantification of CoQ8 in Example 2 (1). Table 8 shows the results.

Table 8

	Cell amount [OD660]	Amount of CoQ10 Accumulated [mg/l]
KY4113/pEG400	23.7	65.2
KY4113/pRSDX-1	23	81
KY4113/pRSDX-2	24.4	81.9
KY4113/pRSYMDX1	25.8	117.9

The amount of CoQ10 produced was significantly higher in KY4113/pRSDX-1, KY4113/pRSDX-2 and KY4113/pRSYMDX1 than in the control strain KY4113/pEG400.

#### Example 8 Determination of the Activity of the Enzyme Encoded by *yaeM* Gene

##### (1) Overexpression of *yaeM* Gene

A recombinant plasmid that can express *yaeM* gene sufficiently was constructed using PCR [Science, 230, 1350 (1985)], as follows.

A sense primer having a nucleotide sequence of SEQ ID NO:24 and an antisense primer having a nucleotide sequence of SEQ ID NO:25 were synthesized using a DNA synthesizer.

A restriction enzyme BamH I site was added to each of 5'-ends of the sense and antisense primers. *yaeM* gene was amplified by PCR using chromosomal DNA of *E.coli* as a template, these primers, Taq DNA polymerase (Boehringer), and DNA Thermal cycler (Perkin Elmer Japan).

PCR was carried out by 30 cycles, one cycle consisting of reaction at 94°C for 30 seconds, reaction at 55°C for 30 seconds, and reaction at 72°C for 2 minutes followed by reaction at 72°C for 7 minutes.

The amplified DNA fragments and pUC118 (TAKARA SHUZO Co., Ltd.) were digested with a restriction enzyme BamH I, then each DNA fragment was purified by agarose gel electrophoresis.

Both purified fragments were mixed together, then treated with ethanol, allowing DNA to precipitate. The resultant DNA precipitate was dissolved in 5  $\mu$ l of distilled water for ligation reaction to occur, thereby obtaining recombinant DNA.

The recombinant DNA was confirmed to be *yaeM* gene by determining its DNA sequence.

Plasmids were extracted from the microorganism having the recombinant DNA, digested with a restriction enzyme BamH I, and subjected to agarose gel electrophoresis, thereby obtaining DNA

fragments containing BamH I-treated yaeM gene.

pQE30 (Qiagen, Inc) was digested with a restriction enzyme BamH I, then subjected to agarose gel electrophoresis, thereby obtaining BamH I-treated pQE30 fragments.

The resultant DNA fragments containing BamH I-treated yaeM gene were mixed with BamH I-digested pQE30 fragments, and treated with ethanol for DNA to precipitate. The DNA precipitate was dissolved in 5  $\mu$ l of distilled water for ligation reaction to occur, thereby obtaining recombinant DNA.

*E. coli* JM109 was transformed using the recombinant DNA by standard techniques. Then the transformants were spread on LB agar medium containing 100  $\mu$ g/ml of ampicillin, then cultured overnight at 37°C.

Plasmids were isolated from the *E.coli* in the same manner as described above.

Similarly, the isolated plasmid was cleaved with various restriction enzymes to examine the structure, then the nucleotide sequence was determined, thereby confirming the plasmids contained the DNA fragments of interest. The plasmid was named pQEDXR.

## (2) Determination of Activity of yaeM Gene Product

### ① Purification of yaeM gene product

The pQEDXR constructed in (1) was introduced into *E.coli* M15 (Qiagen, Inc) having pREP4 by standard techniques, and a strain M15/pREP4+pQEDXR resistant to 200  $\mu$ g/ml of ampicillin and 25  $\mu$ g/ml of kanamycin was obtained.

The strain M15/pREP4+pQEDXR was cultured at 37°C in 100 ml of LB liquid medium containing 200  $\mu$ g/ml of ampicillin and 25  $\mu$ g/ml of kanamycin. When the turbidity at 660nm reached 0.8, isopropyl thiogalactoside was added to a final concentration of 0.2 mol/l. Subsequently, the strain was cultured for 5 hours at 37°C, then the supernatant of the culture was removed by centrifugation (3000 rpm, 10 minutes). The cells were suspended in 6 ml of 100 mol/l Tris-hydrochloric acid buffer (pH 8.0), then disrupted using a ultrasonicator (SONIFIER, BRANSON) while cooling with ice. The obtained cell-disrupted solution was centrifuged at 10,000 rpm for 20 minutes at 4°C, thereby collecting the supernatant. The supernatant centrifuged from the cellular extract was introduced into a Ni-NTA resin column (Qiagen, Inc), then washed with 20 ml of a washing buffer (100 mol/l Tris-hydrochloric acid (pH 8.0), 50 mol/l imidazole, 0.5 % Tween 20). Then 10 ml of an elution buffer (100 mol/l Tris-hydrochloric acid (pH 8.0), 200 mol/l imidazole) was introduced into the column,

thus fractionating the eluate into 1 ml each.

Protein amounts for each fraction were measured using a kit for quantifying protein amount (Bio-Rad Laboratories), thus obtaining a fraction containing proteins as a purified protein fraction.

## ② Preparation of a Substrate, 1-deoxy-D-xylulose 5-phosphate

A reaction substrate, 1-deoxy-D-xylulose 5-phosphate was prepared as described below. 1-Deoxy-D-xylulose 5-phosphate was detected by measuring the absorbance at 195 nm using HPLC [Column: Senshu pak NH2-1251-N (4.6 x 250 mm, Senshu), mobile phase: 100 mol/l  $\text{KH}_2\text{PO}_4$  (pH 3.5)].

The plasmid pQDXS-1 that allows overexpression of *E.coli* dxs gene was introduced into *E.coli* M15/pREP4 in the same manner as described above, obtaining a strain M15/pREP4+pQDXS-1.

This strain was cultured in the same way as in Example 8 (2)①, then dxs protein was purified using Ni-NTA resin column.

The purified dxs protein was added to a 20 ml of reaction solution [100 mol/l Tris- hydrochloric acid (pH 7.5), 10 mol/l sodium pyruvate, 30mol/l DL-glyceraldehyde-3-phosphate, 1.5 mol/l thiamine pyruvate, 10 mol/l  $\text{MgCl}_2$ , 1mol/l DL-dithiothreitol] then maintained at 37°C.

After reacting for 12 hours, the reaction solution was diluted with water to 300 ml, introduced into an activated carbon column (2.2 x 8 cm) followed by a Dowex 1-X8 (C1-type, 3.5 x 25 cm), then eluted with 1 % saline solution. After the eluted fraction was concentrated, the fraction was introduced into Sephadex G-10 (1.8 x 100 cm), then eluted with water. Finally fractions containing 1-deoxy-D-xylulose 5-phosphate were freeze-dried, thereby obtaining about 50 mg of white powder.

This powder was confirmed to be 1-deoxy-D-xylulose 5-phosphate by NMR analysis (A-500, JEOL Ltd.).

## ③ Determination of Enzymatic Activity of yaeM Gene Product

0.3 mol/l of 1-deoxy-D-xylulose 5-phosphate (final concentration) synthesized as described above was added to 1 ml of a reaction solution containing 100 mol/l Tris-hydrochloric acid (pH 7.5), 1 mol/l  $\text{MnCl}_2$ , 0.3 mol/l NADPH and yaeM gene product obtained in Example 8 (2)①, and then incubated at 37°C. The increase and decrease in NADPH during incubation was traced by reading the absorbance at 340 nm using a spectrophotometer (UV-160, SHIMADZU CORP.), suggesting that NADPH decreased with time.

To confirm the structure of the reaction product, the reaction was carried out similarly, but on a larger scale, thus isolating the product. 200 ml of a reaction solution with a composition the same as that described above except that the concentration of 1-deoxy-D-xylulose 5-phosphate was 0.15mol/l, was incubated for 30 minutes at 37°C. Then the whole amount of the reaction solution was added to an activated carbon column, diluted with water to 1L, then added to a Dowex 1-X8 (C1-type, 3.5 x 20 cm) column.

The solution was eluted with 400 ml of 1 % saline solution, added to a Sephadex G-10 (1.8 x 100 cm), then eluted with water. The eluted fraction was freeze-dried, thereby isolating the reaction product.

The molecular formula of the reaction product isolated from HR-FABMS analysis was assumed to be  $C_5H_{12}O_5P$  [m/z 215.0276 (M-H)<sup>-</sup>,  $\Delta$  -4.5 mmu]. NMR analysis for <sup>1</sup>H and <sup>13</sup>C resulted in the following chemical shifts.

<sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz):  $\delta$  4.03 (ddd, J = 11.5, 6.5, 2.5 Hz, 1H), 3.84 (ddd, J = 11.5, 8.0, 6.5 Hz, 1H), 3.78 (dd, J = 8.0, 2.5 Hz, 1H), 3.60 (d, J = 12.0 Hz, 1H), 3.50 (d, J = 12.0 Hz, 1H), 1.15 (s, 3H); <sup>13</sup>C NMR (D<sub>2</sub>O, 125 MHz):  $\delta$  75.1 (C-2), 74.8 (C-3), 67.4 (C-1), 65.9 (C-4), 19.4 (2-Me)

The chemical shifts resulted from NMR analysis for <sup>1</sup>H and <sup>13</sup>C of compounds obtained by treating the reaction products with alkaline phosphatase (TAKARA SHUZO CO., LTD.) were completely identical with that resulted from NMR analysis of 2-C-methyl-D-erythritol synthesized in the method described in Tetrahedron Letter, 38, 6184 (1997).

Further the angle of rotation of the former compound was  $[\alpha]_D^{21} = +6.0$  (c = 0.050, H<sub>2</sub>O), identical with the angle of rotation  $[\alpha]_D^{25} = +7.0$  (c = 0.13, H<sub>2</sub>O) of 2-C-methyl-D-erythritol, reported in Tetrahedron Letter, 38, 6184 (1997).

These results reveal that the reaction product of yaeM gene product was 2-C-methyl-D-erythritol 4- phosphate. That is, yaeM gene product was found to have activity to yield 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate with consumption of NADPH. Based on this catalytic activity, this enzyme was named 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

#### ④ Characteristics of 1-deoxy-D-xylulose 5-phosphate reductoisomerase

The enzymological characteristics of 1-deoxy-D-xylulose 5-phosphate reductoisomerase were examined using 1 ml of the reaction system as described in Example 8 (2) ③. Here, 1 unit is defined

as the activity to oxidize 1 mmol of NADPH per a minute.

The activity decreased below 1/100 when NADPH was replaced with NADH.

No reaction occurred when 1-deoxy-D-xylulose was used instead of 1-deoxy-D-xylulose 5-phosphate.

SDS-PAGE analysis showed that this enzyme was consisted of 42 kDa polypeptide.

Table 9 shows effect on the reaction system by the addition of metals.

Table 9

Effect of various metal ions on the activity of

1-deoxy-D-xylulose 5-phosphate reductoisomerase

Additives	Specific Activity (units / mg protein)
none	0.3
EDTA	N.D.
MnCl <sub>2</sub>	11.8
CoCl <sub>2</sub>	6.0
MgCl <sub>2</sub>	4.0
CaCl <sub>2</sub>	0.2
NiSO <sub>4</sub>	0.2
ZnSO <sub>4</sub>	0.3
CuSO <sub>4</sub>	N.D.
FeSO <sub>4</sub>	N.D.

These metal ions and EDTA were added such that the concentration of each was 1mol/l. N.D. indicates that no activity was detected.

K<sub>m</sub> for 1-deoxy-D-xylulose 5-phosphate and NADP in the presence of MnCl<sub>2</sub> were 249 μmol/l and 7.4 μmol/l, respectively.

Figure 1 shows the effect of reaction temperature and Figure 2 shows the effect of reaction pH.

#### Example 9 Construction and Characteristics of yaeM - deleted mutant

##### (1) Construction of yaeM - disrupted mutant

To test whether 1-deoxy-D-xylulose 5-phosphate reductoisomerase is essential for cell growth or not, a 1-deoxy-D-xylulose 5-phosphate reductoisomerase-deleted mutant was constructed as described below.

A kanamycin-resistant gene cassette for insertion into yaeM gene was produced as described below.

The plasmid pMEW41 obtained in Example 1 (2) ② was digested with a restriction enzyme Bal

I, and was subjected to agarose gel electrophoresis, thereby obtaining a Bal I– treated DNA fragment.

Tn5 was digested with restriction enzymes Hind III and Sam I, then the both ends were blunt-ended using a DNA blunting kit (TAKARA SHUZO CO., LTD.).

The resultant blunt-ended DNA fragments were mixed with previously obtained Bal I-treated pMEW41DNA fragments, and then the mixture was treated with ethanol. Next the obtained DNA precipitate was dissolved into 5  $\mu$ l of distilled water for ligation reaction to occur, thereby obtaining recombinant DNA.

*E. coli* JM109 (purchased from TAKARA SHUZO CO., LTD.) was transformed using this recombinant DNA according to standard techniques. Next the transformant was spread on LB agar medium containing 100  $\mu$ g/ml of ampicillin and 15  $\mu$ g/ml of kanamycin, then cultured overnight at 37°C.

Several ampicillin-resistant transformant colonies grown on the medium were shake-cultured for 16 hours at 37°C in 10 ml of LB liquid medium containing 100  $\mu$ g/ml of ampicillin and 15  $\mu$ g/ml of kanamycin.

The resulting culture was centrifuged to collect cells.

Plasmids were isolated from the cells according to the standard techniques.

The plasmids isolated as described above were cleaved with various restriction enzymes to test their structure. As a result, the plasmid was confirmed to contain the DNA fragment of interest and was named pMEW41Km.

yacM gene on a chromosomal DNA of *E.coli* was disrupted by homologous recombination using pMEW41Km. Figure 3 shows the schematic diagram for this recombination.

pMEW41Km was digested with restriction enzymes Hind III and Sac I, subjected to agarose gel electrophoresis, thus purifying linear fragments. *E.coli* FS1576 was transformed using the fragments according to standard techniques. The strain FS1576 is available as the strain ME9019 from National Institute of Genetics. The transformants were spread on LB agar medium containing 15  $\mu$ g/ml of kanamycin and 1 g/l of 2-C-methyl-D-erythritol, then cultured overnight at 37°C.

Several kanamycin-resistant colonies that grew on the medium were shake-cultured for 16 hours at 37°C in 10 ml of LB liquid medium containing 15  $\mu$ g/ml of kanamycin and 1 g/l of 2-C-methyl-D-erythritol.



The resulting culture was centrifuged to collect cells.

Chromosomal DNA was isolated from the cells by the standard techniques.

The chromosomal DNA was digested with a restriction enzyme Sma I or Pst I. Chromosomal DNA of the strain FS1576 was digested with a restriction enzyme in the same way. These DNAs digested with restriction enzymes were subjected to agarose gel electrophoresis by the standard techniques, and then to Southern hybridization analysis using the kanamycin-resistant gene and *yaem* gene as probes. Therefore, it was confirmed that the chromosomal DNA of the kanamycin-resistant colonies had a structure as shown in Fig. 3, that is, *yaem* gene was disrupted by the kanamycin-resistant gene.

## (2) Characteristics of *yaem*-disrupted mutant

The *yaem*-disrupted strain produced as described above and its parent strain FS1576 were spread on LB agar medium and the same medium containing 1 g/l of 2-C-methyl-D-erythritol, then cultured at 37°C. Table 10 shows the cell growth after 2 days of culture.

Table 10  
Effect of deletion of *yaem* gene on the *E.coli* growth

Strain	Cell growth on each medium * <sup>1</sup>	
	LB	LB + ME * <sup>2</sup>
FS1576	+	+
<i>yaem</i> -deleted strain	-	+

\*1: Cell growth (+ indicates good growth; - indicates no growth)

\*2: ME indicates the addition of 1 g/l of 2-C-methyl-D-erythritol.

No *yaem*-deleted mutants grew on a medium lacking 2-C-methyl-D-erythritol. Therefore, This gene was shown to be essential for the cell growth in the absence of 2-C-methyl-D-erythritol.

Example 10 Effect of 1-deoxy-D-xylulose 5-phosphate reductoisomerase inhibitor for cell growth.

The following experiments were conducted based on the assumption that fosmidomycin could inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase because 2-C-methyl-D-erythritol 4-phosphate, a product from 1-deoxy-D-xylulose 5-phosphate reductoisomerase reaction, or reaction intermediates expected to be produced in this enzyme reaction is structurally analogous to fosmidomycin.

In the presence of fosmidomycin, the activity 1-deoxy-D-xylulose 5-phosphate reductoisomerase was measured by the method as described in Example 8 in order to examine the effect on the enzymatic

activity.

Fosmidomycin had been synthesized according to the method described in Chem. Pharm. Bull., 30, 111-118 (1982).

Total volume of reaction solution was reduced to 0.2 ml from the volume of reaction solution described in Example 8 (2), but each concentration was kept at the same level as the system of Example 8 (3). Fosmidomycin at various concentration was added to the reaction solution, then the reaction was carried out at 37°C. The increase and decrease in NADPH were measured using Bench mark micro plate reader (Bio-Rad Laboratories).

As shown in Fig. 4, fosmidomycin was shown to inhibit 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

*E. coli* W3110 was spread on LB agar medium, the same medium containing 3.13 mg/l of fosmidomycin, and the same medium containing 3.13 mg/l of fosmidomycin and 0.25 g/l of 2-C-methyl-D-erythritol, then cultured at 37°C.

Two days after culturing, the microorganism could grow on the two types of media, that is, the LB agar medium and the same medium containing fosmidomycin and 0.25 g/l of 2-C-methyl-D-erythritol, but no microorganism grew on the LB agar medium supplemented only with fosmidomycin.

These results clearly shows that fosmidomycin inhibited the cell growth by inhibiting 1-deoxy-D-xylulose 5-phosphate reductoisomerase. Accordingly, a substance inhibiting yaeM gene product (1-deoxy-D-xylulose 5-phosphate reductoisomerase) activity can be an effective antibiotic agent or herbicide.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

#### Industrial Applicability

The present invention can provide a process for producing isoprenoid compounds comprising integrating DNA into a vector wherein the DNA contains one or more DNA involved in biosynthesis of isoprenoid compounds useful in pharmaceuticals for cardiac diseases, osteoporosis, homeostasis, prevention of cancer, and immunopotential, health food and anti-fouling paint products against

barnacles, introducing the resultant recombinant DNA into a host cell derived from prokaryote, culturing the obtained transformants in a medium, allowing the transformant to produce and accumulate isoprenoid compounds in the culture, and recovering the isoprenoid compounds from the culture; a process for producing a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds comprising integrating DNA containing one or more DNA encoding the protein into a vector, introducing the resultant recombinant DNA into a host cell, culturing the obtained transformant in a medium, allowing the transformant to produce and accumulate said protein in the culture, and recovering said protein from the culture; the protein; and novel enzymatic protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate; and a method for screening a compound with antibiotic and/or weeding activity comprising screening a substance inhibiting the enzyme.

#### Sequence Listing Free Text

SEQ ID NO: 12: synthetic DNA

SEQ ID NO: 13: synthetic DNA

SEQ ID NO: 14: synthetic DNA

SEQ ID NO: 15: synthetic DNA

SEQ ID NO: 16: synthetic DNA

SEQ ID NO: 17: synthetic DNA

SEQ ID NO: 18: synthetic DNA

SEQ ID NO: 19: synthetic DNA

SEQ ID NO: 20: synthetic DNA

SEQ ID NO: 21: synthetic DNA

SEQ ID NO: 22: synthetic DNA

SEQ ID NO: 23: synthetic DNA

SEQ ID NO: 24: synthetic DNA

SEQ ID NO: 25: synthetic DNA

SEQ ID NO: 32: synthetic DNA

SEQ ID NO: 33: synthetic DNA



## CLAIMS

1. A process for producing isoprenoid compounds comprising integrating DNA into a vector wherein the DNA contains one or more DNA selected from the following (a), (b), (c), (d), (e) and (f):

- (a) a DNA encoding a protein having activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate,
- (b) a DNA encoding farnesyl pyrophosphate synthase,
- (c) a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:3, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 3 and has activity to improve efficiency in the biosynthesis of isoprenoid compounds,
- (d) a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:4, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 4 and has activity to improve efficiency in the biosynthesis of isoprenoid compounds,
- (e) a DNA encoding a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate, and
- (f) a DNA encoding a protein that can hybridize under stringent conditions with DNA selected from (a), (b), (c), (d) and (e), and has activity substantially identical with that of the protein encoded by the selected DNA;

introducing the resultant recombinant DNA into a host cell derived from prokaryotes, culturing the obtained transformant in a medium; allowing the transformant to produce and accumulate isoprenoid compounds in the culture; and recovering the isoprenoid compounds from the culture.

2. The process according to claim 1, wherein the DNA encoding a protein having activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate is a DNA encoding a protein that has an amino acid sequence of any one of SEQ ID NOS: 1, 26 and 28, or a DNA encoding a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1, 26 or 28 and has activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and

glyceraldehyde 3-phosphate.

3. The process according to claim 1 or 2, wherein the DNA has a nucleotide sequence of any one of SEQ ID NOS: 6, 27 and 29.
4. The process according to claim 1, wherein a DNA encoding farnesyl pyrophosphate synthase is a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:2, or a DNA encoding farnesyl pyrophosphate synthase that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 2.
5. The process according to claim 1 or 4, wherein the DNA has a nucleotide sequence of SEQ ID NO:7.
6. The process according to claim 1, wherein the DNA encoding a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate is a DNA encoding a protein that has an amino acid sequence of SEQ ID NO:5 or 30, or a DNA encoding a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5 or 30 and has activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.
7. The process according to claim 1 or 6, wherein the DNA has a nucleotide sequence of SEQ ID NO:10 or 31.
8. The process according to claim 1, wherein the DNA has a nucleotide sequence of SEQ ID NO:8 or 9.
9. The process according to claim 1, wherein the isoprenoid compound is one selected from ubiquinone, vitamin K<sub>2</sub> and carotenoids.
10. A protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds and selected from the following (a), (b) and (c):
  - (a) a protein having an amino acid sequence of SEQ ID NO:3, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 3,
  - (b) a protein having an amino acid sequence of SEQ ID NO:4, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 4, and

- (c) a protein having an amino acid sequence of SEQ ID NO:5, or a protein having an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5.

11. A process for producing a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds comprising integrating DNA encoding the protein of claim 10 into a vector, introducing the resultant recombinant DNA into a host cell, culturing the obtained transformant in a medium, allowing the transformant to produce and accumulate said protein in the culture, and recovering said protein from the culture.

12. The process according to claim 1 or 11, wherein the transformant is a microorganism belonging to the genus *Escherichia*, *Rhodobacter* or *Erwinia*.

13. A DNA encoding a protein having activity to improve efficiency in the biosynthesis of isoprenoid compounds and described in any one of the following (a) to (g):

- (a) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:3,
- (b) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:4,
- (c) a DNA encoding a protein having an amino acid sequence of SEQ ID NO:5,
- (d) a DNA having a nucleotide sequence of SEQ ID NO:8,
- (e) a DNA having a nucleotide sequence of SEQ ID NO:9,
- (f) a DNA having a nucleotide sequence of SEQ ID NO:10, and
- (g) a DNA which can hybridize with any one of DNA of (a) to (f) under stringent conditions.

14. A method for screening a substance having antibiotic activity comprising screening a substance that inhibits the reaction of a protein having activity of an enzyme selected from those present on the non-mevalonate pathway in which 1-deoxy-D-xylulose 5-phosphate biosynthesized from pyruvic acid and glyceraldehyde 3-phosphate is converted to 2-C-methyl-D-erythritol 4-phosphate from which isopentenyl pyrophosphate is biosynthesized.

15. A method for screening a substance having weeding activity comprising screening a substance that inhibits the reaction of a protein having activity of an enzyme selected from those present on the non-mevalonate pathway in which 1-deoxy-D-xylulose 5-phosphate biosynthesized from pyruvic acid and glyceraldehyde 3-phosphate is converted to 2-C-methyl-D-erythritol 4-phosphate from which isopentenyl pyrophosphate is biosynthesized.

16. The screening method according to claim 14 or 15, wherein the protein is a protein described in the following (a) or (b):

- (a) a protein having activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate, or
- (b) a protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.

17. The screening method according to claim 16, wherein the protein that catalyzes a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate is a protein that has an amino acid sequence of SEQ ID NO: 1, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 1 and has activity to catalyze a reaction to produce 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and glyceraldehyde 3-phosphate.

18. The screening method according to claim 16, wherein the protein having activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate is a protein that has an amino acid sequence of SEQ ID NO: 5, or a protein that has an amino acid sequence wherein one to several amino acid residues are deleted, substituted or added in the amino acid sequence of SEQ ID NO: 5 and has activity to catalyze a reaction to produce 2-C-methyl-D-erythritol 4-phosphate from 1-deoxy-D-xylulose 5-phosphate.

19. A substance having antibiotic activity, which is obtained by the screening method according to claim 14.

20. A substance having weeding activity, which is obtained by the screening method according to claim 15.

21. An antibiotic agent comprising the substance of claim 19.

22. A herbicide comprising the substance of claim 20.



## ABSTRACT

The present invention provides a process for producing isoprenoid compounds or proteins encoded by DNA using DNA that contains one or more of the DNA encoding proteins having activity to improve efficiency in the biosynthesis of isoprenoid compounds effective in pharmaceuticals for cardiac diseases, osteoporosis, homeostasis, prevention of cancer, and immunopotentiality, health food and anti-fouling paint products against barnacles; the DNA; the protein; and a method for screening a substance with antibiotic and weeding activities comprising screening a substance inhibiting enzymatic reaction on the non-mevalonate pathway.

Fig. 1

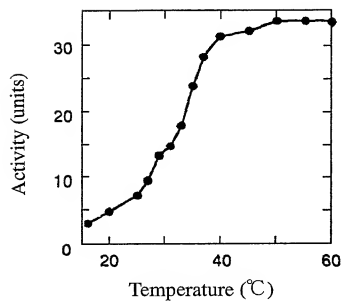


Fig. 2

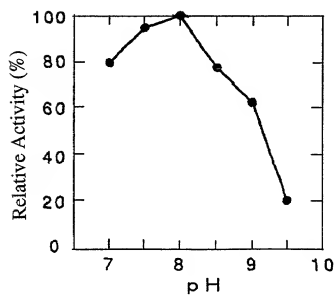


Fig. 3

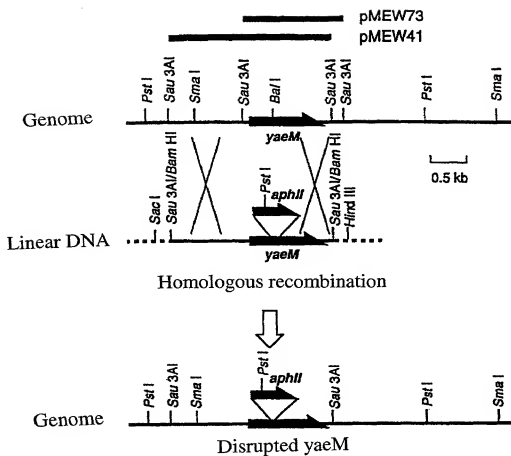
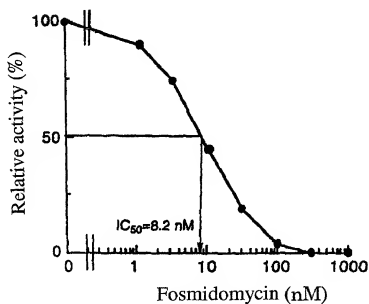


Fig. 4



Attorney's Docket No.: \_\_\_\_\_

**DECLARATION, POWER OF ATTORNEY AND PETITION**

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

A PROCESS FOR PRODUCING ISOPRENOID COMPOUNDS BY MICROORGANISMS  
AND A METHOD FOR SCREENING COMPOUNDS WITH ANTIBIOTIC OR WEEDING

the specification of which

ACTIVITY

☐ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and amended on \_\_\_\_\_.

☒ was filed as PCT international application

Number PCT/JP99/01987

on April 14, 1999

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
<u>103101/1998</u>	<u>Japan</u>	<u>April 14, 1998</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>221910/1998</u>	<u>Japan</u>	<u>August 5, 1998</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
<u>035739/1999</u>	<u>Japan</u>	<u>February 15, 1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

\_\_\_\_\_  
(Application Number)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Number)

\_\_\_\_\_  
(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
<hr/>	<hr/>	<hr/>
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<hr/>	<hr/>	<hr/>

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I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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